

Ectomycorrhizal Fungi Facilitate Competitive Interactions Between Tree Taxa:

Host Preference, Seedling Recruitment, and Forest Succession

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor of Philosophy
in the Department of Biology in the Graduate School
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ABSTRACT

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Abstract

The mycorrhizal mutualism is one of the earliest and most influential of all terrestrial symbioses. As the primary method used by most plants to acquire nutrients from the soil, mycorrhizal fungi help to shape the structure and composition of many ecosystems. Ectomycorrhizal (EM) fungi play an especially significant role because most EM fungi prefer a limited number of host taxa, and EM plant species likewise associate with a select assemblage of the available EM fungi. This host preference issue, combined with the high diversity of EM fungi in forest ecosystems, complicates interspecies competition both among fungi and among plants, because these plant and fungal communities interact.

Despite recent attempts at documenting mycorrhizal fungi in the context of ecological succession, many questions remain about the underlying causal relationships among EM fungi, soil conditions, and plant community assembly. The succession of mycorrhizal fungi often mirrors the succession of plants, and ectomycorrhizal (EM) community composition may affect the outcome of competition among trees during succession. In a pine-oak seral system, I tested the ability of *Pinus taeda* and *Quercus alba* seedlings to associate with EM fungi when planted under both conspecific and heterospecific adults. I found that EM communities under pine and oak canopy were distinct regardless of seedling identity, indicating that the fungal associations of adult

trees determine which EM species are available in the soil. In addition, pine seedlings planted under oak canopy showed decreased mycorrhization and growth compared to those planted under pine canopy, while oak seedlings showed no negative effects of heterospecific planting. This impaired ability of pine seedlings to associate with the EM community established under oaks may deter pine recruitment and facilitate the late-seral replacement of pines with oaks.

While EM fungal communities correlate with the dominant species of host tree, soil properties do as well, making it difficult to establish causality among these three variables. Soil was collected from oak- and pine-dominated stands and dried to kill off mature mycelium, leaving only the spore bank as a source of inoculum for pine and oak seedlings. EM root tips were collected for molecular identification of fungal species based on ITS barcoding, and soil samples from field and laboratory conditions were analyzed for fungal diversity using 454 sequencing. I found a reduced influence of canopy type and a more pronounced influence of seedling identity when compared to the EM communities on seedlings planted in the field, suggesting that adult trees do alter the availability of fungi by directly promoting the growth of their preferred EM associates.

The availability of EM fungi can also affect seedlings at the interface between EM- an AM-dominated forest. *Dicymbe corymbosa* is a neotropical, EM-associated tree that forms monodominant stands nested within a diverse matrix of AM-associated taxa.

I tested the hypothesis that seedlings of *D. corymbosa* which recruit outside of monodominant stands have limited access to EM symbionts compared with those which recruit inside *D. corymbosa* stands. EM root tips and rhizosphere soil were collected from seedlings along two transects inside monodominant stands and three transects across the transition zone into mixed forest. Seedlings inside monodominant stands yielded both a greater quantity of mycorrhized root tips and a higher diversity of EM species than transition zone seedlings. Of the fungal families commonly found on adult roots, the Boletaceae were notably underrepresented on all seedlings. In the transition zones, high-throughput sequencing of soil also detected a decrease in EM diversity with distance from the parent tree.

Seedlings of *D. corymbosa* may benefit from recruiting within monodominant stands by tapping into common mycorrhizal networks (CMNs) to acquire low-cost nitrogen and, potentially, photosynthates produced by conspecific adults. Leaves of stand adults, stand seedlings, and mixed-forest seedlings were collected for stable isotope analysis to track the transfer of nitrogen and carbon through CMNs. The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ results contradicted each other, suggesting that more complicated interactions may be playing out among adults, seedlings, and fungi.

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1. White oaks suppress seedling recruitment of loblolly pines via belowground ectomycorrhizal networks

1.1 Introduction

Ecological succession is the product of numerous interwoven processes that generate complex responses in one another. Because of this complexity, it has often proved difficult to identify causal relationships among the biotic and abiotic factors involved. A wide variety of mechanisms have been implicated in the regulation of succession, including environmental variables (Oosting 1948), stochastic processes (Horn 1975), disturbance regimes (Pickett 1976), resource availability (Tilman 1985), and interspecies competition (Huston and Smith 1987).

More recently, the relationship between aboveground and belowground communities has been emphasized (Bardgett et al. 2005). Several interactions of this type have been proposed as drivers of succession, including mycorrhizal fungi affecting plant diversity (van der Heijden et al. 1998), host plants as drivers of fungal diversity (Jumpponen et al. 2002), plant traits controlling litter and soil properties (Cornelissen et al. 1999), soil heterogeneity facilitating fungal diversity (Reynolds et al. 2003), and so on. The issue of which biotic and abiotic factors catalyze seral change and which merely respond to it remains unresolved.

The succession of mycorrhizal fungi reflects the succession of their mycorrhizal host plants (Allen 1991, Helm 1996), and many of the classic systems used to study

succession in plants have more recently been utilized for examining belowground fungal succession (i.e. Cazares et al. 2005, Horton et al. 2005, Ishida et al. 2008, Piotrowski et al. 2008). In the Piedmont region of North Carolina, mycorrhizal succession (Fig. 1) can be divided into three stages: primary succession of arbuscular mycorrhizal (AM) fungi associating with grasses and herbaceous plants, replacement of AM with mid-seral ectomycorrhizal (EM) species associating with pine forest, and shifts in the EM species assembly to reach a late-seral community composition associating with oak-hickory forest. Species richness of EM fungi increases along with the diversity of host plants during late succession (Jumpponen et al. 2002), and some mid-seral EM species may be replaced by late-seral species (Deacon and Fleming 1992).

EM fungi are generally considered to have low host-specificity (Molina et al. 1992, Horton and Bruns 2001), such that community compositions of plants and fungi should have little effect on one another. However, there is also evidence that some degree of host preference is widely prevalent in EM-dominated ecosystems (Smith et al. 2009), such as temperate forests. A fungal species exhibiting host preference may proliferate when associated with a given host species, and may be present at lower abundances or absent when preferred hosts are not available. In addition to affecting fungal communities, host preference plays an important role in plant community assembly (Tedersoo et al. 2008, Smith et al. 2009). Some EM fungi previously considered to be host-generalists may actually confer unequal benefits to different tree species,

promoting the growth of one species over another, even when those fungi freely associate with both hosts (Pande et al. 2007).

In the Piedmont region of North Carolina, old-field succession begins with native grasses and herbaceous plants dominating for the first 3 years; then pines overtop the grasses by 5 years and form closed stands in 10-15 years; lastly, oaks and hickories begin replacing the pines by 70-80 years and attain dominance in 150-200 years (Oosting 1942). Both oak and pine are ectomycorrhizal hosts, and a number of preferential associations have been observed from fruit bodies, such as the association of the *Suillus-Rhizopogon* lineage with Pinaceae (Smith and Thiers 1964, Molina et al. 1992).

When tree seedlings are first establishing, they may preferentially associate with older fungal individuals in order to take advantage of common mycorrhizal networks (CMNs) (Simard and Durall 2004). These mature fungi come "prepaid" by an adult host, of either the same or a different tree species, and may be able to provide low-cost nutrients to the seedlings. In some systems, CMNs are critical for seedling survival because they compensate for the competitive effects of adult conspecifics (Booth and Hoeksema 2010). However, CMNs can also have a variety of effects on seedlings depending on which species of hosts and fungi are involved (Nara 2006).

The objective of this study was to determine the effects of EM community composition on recruitment of conspecific and heterospecific seedlings in a pine-oak seral system. The dominant canopy species in each seral stage should select for its

preferred set of fungal associates, which in turn limits the composition of EM fungi readily available for seedling associations. I hypothesized that EM fungal communities under oak canopy would be more diverse as well as compositionally distinct from EM fungal communities under pine canopy. Given the general pattern of oaks succeeding pines, I predicted that oak seedlings would readily associate with the fungi available under pine canopy, while pine seedlings would have difficulty associating with the fungi available under oak canopy.

1.2 Methods

1.2.1 Study site

The Duke Forest, located in Durham, North Carolina, was established in 1931 as an outdoor laboratory for foresters and ecologists. The original five thousand acres were a mosaic of local land-use history, including cultivated fields, recent fallow fields, and fields abandoned anywhere from a decade to a century prior, as well as riparian zones, bottomlands, and rocky ridges that had never been cleared (Oosting 1942). Ten paired plots were established across different sites within the Duke Forest, with each pair consisting of one plot in a stand dominated by white oak (*Quercus alba*), and the other plot in a stand dominated by loblolly pine (*Pinus taeda*) immediately adjacent to the oak stand. Eight of these plots were in the Durham Division and two were in the Korstian Division. While most sites have retained the same cover type as when they were first acquired, two of the plots now dominated by white oak had a mixed loblolly-oak cover

type in 1931. All plots were on deep to very deep and moderately well drained to well drained soils, predominantly Carolina Slate derived silt loams with one plot on igneous intrusive derived sandy loam.

1.2.2 Experimental design and sampling

I conducted a reciprocal transplant experiment using loblolly pine and white oak seedlings, both of local wild-type genetic stock. Seeds were germinated in trays of autoclaved perlite in the laboratory and grown for approximately three months prior to transplanting. For comparison, seedlings of both species were also planted under conspecific adults, for a total of ten pine and ten oak seedlings in each plot. This made for a total of four treatment types: oak seedlings under oak canopy, oak seedlings under pine canopy, pine seedlings under oak canopy, and pine seedlings under pine canopy. Seedlings were planted in Fall 2010 and harvested in Spring 2011. EM root tips were harvested from all seedlings, including multiple representatives of all visible morphotypes. In total, 81 oak seedlings and 89 pine seedlings yielded sequences of EM fungi from their roots. For pine seedlings from four of the ten plots, I also counted the number of mycorrhizal root clusters per centimeter of root (mycorrhization) and measured new shoot growth at the time of harvest. These measurements were not attempted with oak seedlings because the highly branched fine roots and small mycorrhizal tips rendered a quantitative comparison impractical (Fig. 2). Samples of O-

horizon and A-horizon soils were collected from all ten plots and analyzed for moisture, pH, carbon and nitrogen content, and texture.

Fungi were identified by extracting DNA from EM root tips and sequencing the internal transcribed spacer (ITS) region of nuclear ribosomal DNA, the universal DNA barcode for fungi (Schoch et al. 2012). DNA was extracted following the Extract-N-Amp protocol (Sigma-Aldrich, Inc.) with a single root tip in 20µl extraction solution. The ITS region was amplified by polymerase chain reaction using the fungal-specific forward primer ITS1-F (Gardes and Bruns 1993) and either the reverse primer ITS4 (White et al. 1990) or the reverse primer ITS2 (White et al. 1990). Capillary sequencing of ITS fragments was conducted using Big Dye v3.1 and visualized on a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA). Sequences were submitted to Genbank under accession numbers KF476744 - KF477091.

1.2.3 Data analysis

Sequences were manually edited and assembled at 100% similarity in Sequencher 4.5 (Gene Codes, Ann Arbor, MI) to check for duplicate haplotypes. If a haplotype was sequenced multiple times from the same seedling, these root tips were considered pseudoreplicates and the sequence was recorded only once in the database; for the purpose of data analysis, each seedling was treated as one sampling replicate. Of approximately 1000 root tips picked, around 600 usable EM sequences were recovered;

361 were retained in the final dataset after pseudoreplicates were removed. Sequences were initially assembled at 97% similarity to assign them to operational taxonomic units (OTUs), approximating species-level delineations. If a genus did not segregate into robust OTUs at 97%, other similarity cutoffs were explored. Sequences were then compared against GenBank accessions using the BLASTn algorithm to assign genus- and, when possible, species-level identifications to each OTU.

Soil samples were acquired from within each plot using a 2-cm diameter soil probe. Four cores were taken per plot to a depth of 8-10 cm, the O- and A-horizons separated, and the cores bulked by plot. Subsamples were weighed and oven-dried overnight for measuring water content, while the rest was air-dried for a minimum of one week and then passed through a 2-mm sieve to remove rocks and debris. Soil texture was determined following the pipette method (Gee and Or 2002). For pH measurements, 1.0 g organic or 2.5 g mineral soil was combined with 10 ml or 5 ml deionized water, respectively, and after 15 min a pH probe was inserted. To determine C and N content, subsamples were pulverized in a shatterbox then weighed out into tin capsules containing 9-10 mg organic or 30-40 mg mineral soil for analysis on a Flash EA 1112 Elemental Analyzer (Thermo Scientific, Waltham, MA).

Relative abundances of EM species on different hosts were used as a proxy for host preference. For oak and pine seedlings under each of the two canopy treatments, I estimated fungal diversity per treatment using EstimateS 8.2.0 (Colwell 2009) and

examined the relative similarity of EM communities using nonmetric multidimensional scaling (NMS) and cluster analysis in PC-Ord version 5 (McCune and Mefford 1999).

Nonmetric multidimensional scaling (NMS) is an ordination method well-suited for handling species data because it does not rely on known absences and thus is relatively robust to missing data (McCune and Grace 2002). Species abundances were relativized by species and a three-dimensional solution was chosen based on the stress results of a stepdown procedure. The cluster analysis was computed using the flexible beta joining algorithm (Lance and Williams 1966) at a beta value of -0.25. This method works well for ecological data and yields results similar to Ward's method, which is itself incompatible with Sorensen's distances (McCune and Grace 2002).

1.3 Results

Of the 361 EM sequences, 323 could be assigned to one of 60 OTUs, and the remaining 38 were singletons. Thirteen of these OTUs only appeared on a single seedling species within a single plot, and thus were uninformative for community analysis. The remaining 47 OTUs, however, appeared in multiple plots and/or on both seedling species. The three most abundant fungal species--*Thelephoraceae* sp. 1, *Cenococcum geophilum*, and *Tuber separans*--were found in all four treatments; most other EM species were found at low abundance in only 1-2 treatments (Fig. 3). Fisher's exact

tests are reported for the 29 species that earned a p-value <0.1 associated with either canopy type, seedling identity, or both (Table 1).

Using a three-dimensional solution, NMS accounts for 55% of the variation in the community composition data. The ordination shows EM communities separating by canopy type regardless of seedling identity or geographic distance between sites (Figs. 4, 5). Cluster analysis shows all oak-canopy treatments grouping together; pine-canopy treatments segregate into three groups, with oak and pine seedlings from the same plot clustering together (Fig. 6). While these results are only shown using a three-dimensional ordination and a flexible beta joining algorithm, the grouping together of oak-canopy treatments is robust to ordination dimensionality and this cluster topology is preserved with other joining algorithms such as UPGMA.

Soil properties varied among sites, with oak-canopy plots tending to have lower A-horizon pH, higher clay content, and C:N ratios higher in the A-horizon and lower in the O-horizon when compared to their paired pine plots (Table 2). Several edaphic conditions, including N, C:N ratio, and pH of both the mineral and organic horizons correlated loosely with EM community composition (Fig. 4).

Both pine and oak seedlings were capable of associating with the fungal communities found under the opposite canopy type. However, pine seedlings grown under oak canopy showed significantly less mycorrhization than those grown under

pine canopy ($P < 0.001$). Mycorrhization correlated with growth ($R^2=0.41$, Fig. 7a), which was also significantly less for pines planted under oak ($P < 0.001$).

Due to differences in root morphology, it proved impractical to quantify mycorrhization for oak seedlings. Unlike pines, however, oak seedlings showed no obvious, visible pattern of decreased mycorrhization or growth correlated with heterospecific canopy type (pers. obs.). They also associated with more EM species under both canopy types: with 33 species under oak and 26 species under pine, compared to pine seedlings associating with 20 under oak and 22 under pine. The greater EM species richness of oak seedlings under oak canopy was statistically significant compared with pines under both oak and pine canopy ($P=0.035$ and $P=0.028$ respectively). Using the Chao2 species richness estimator (Chao 1984), oak seedlings under oak canopy hosted the most speciose community (60.6 OTUs) across sites, while oaks and pines under pine had intermediate richness (51.3 and 48.2 respectively), and pine seedlings under oak were relatively depauperate (34).

1.4 Discussion

Most fungal species found in high abundance on the root tips appear to be generalists, since they occur under both canopy types and associate freely with seedlings of both hosts. Overall, EM fungal communities still segregate by canopy type regardless of which seedling species was sampled (Fig. 4), which supports the hypothesis that the

fungi available in the soils for seedlings to associate with are those species actively involved in the EM mutualism and receiving carbon from adult trees (i.e. CMNs). Thus, the preferences of the canopy trees are reflected in the abundances of fungal species found on seedling roots. Cluster analysis (Fig. 6) indicates EM communities under oak canopy are more consistent in their composition across sites, while EM communities from pine plots form three separate clusters. This result suggests that mid-seral EM communities may be substantially influenced by stochastic factors such as founder or priority effects. Late-seral EM communities may assemble in a more deterministic fashion, achieving particular structural similarities despite the variability of the mid-seral communities they replace.

Differences in soil properties may also contribute to the differences in fungal communities, though no single soil property had a strong enough correlation to suggest it might be a driving factor. Most of the properties that correlated with EM community composition (pH, N, and N:C) can be affected by the type of litter deposition (Cornelissen et al. 1999), so EM composition and soil properties may be correlated simply because they are both driven by the dominant canopy species.

According to both observed and estimated species richnesses, oak seedlings planted under oak canopy recovered the highest diversity of EM fungi, indicating that oak-associated fungal communities are more speciose than mid-seral communities even when only one oak species is present. While late-seral plant communities in this region

tend to have a variety of oak and hickory species, sites for this study were chosen specifically to maximize the dominance of a single canopy species (*Q. alba*) so that host diversity would not be a confounding factor. My results contradict the hypothesis that increasing host diversity is the main driver of increasing fungal diversity during late succession (Jumpponen et al. 2002), because higher EM diversity can be seen on white oaks even in the absence of additional host species.

One possible explanation is that loblolly pine associates with a narrower spectrum of EM species than does white oak. Pine seedlings planted under oak canopy yielded the lowest richness of EM species, as well as showing decreased mycorrhization of the roots compared to pine seedlings under pine. These data suggest that pine seedlings recruited under oak canopy may have difficulty finding their ideal mycorrhizal symbionts and/or derive less benefit from the symbionts available in the soil, and so form mycorrhizal root tips with less frequency.

Because the North Carolina Piedmont experiences relatively mild winters and hot summers with periods of limited rainfall, pine seedlings accomplish most of their annual growth in late fall and early spring, after leaf fall and before leaf out (pers. obs.). Thus I expected pine seedlings under a deciduous canopy to grow better than those under a pine-dominated canopy, since the winter light intensity is greater. However, I observed the opposite pattern, indicating that light is not the main factor determining pine seedling growth. Rather, the depressed growth of pine seedlings under oak canopy

correlates with decreased mycorrhization, suggesting that nutrient limitation may impede seedling growth and establishment.

Oak seedlings planted under pine yielded fungal richness similar to that found on their neighboring pine seedlings and showed no signs of difficulty finding suitable EM associates. This result contradicts the previous hypothesis that mid-seral plants use host-specific EM fungi to inhibit the establishment of late-seral seedlings (Kropp and Trappe 1982, Horton et al. 2005). The ability of oak seedlings to associate with a broad range of fungi may actually facilitate recruitment under pine canopy, enabling succession. At the same time, the fungal preferences of adult oaks may also impede the ability of pine seedlings to persist under oak canopy.

The Fisher's exact tests (Table 1) detected not only fungal species with a preference for canopy type but also some fungal species with a preference for seedling identity. Species with a preference for pine canopy were split evenly between Basidiomycetes (including two species of Thelephoraceae and two of *Russula*) and Ascomycetes (including two species of *Tuber* and four Pezizalean fungi). But, with the exception of *Cenococcum geophilum*, species showing a preference for oak canopy belonged entirely to the Basidiomycetes (including two species of *Russula* and two of *Sebacina*).

Only six fungal species showed a distinct preference for seedling host identity. *Tylospora* sp. 1 and *Suillus variegatus* were found on pine seedlings under both canopy

types, indicating they are pine-specific--not a surprising result given the well-known preference of *Suillus* for pines. Two oak-specific species, *Russula* sp. 7 and *Cortinarius livido-ochraceus*, were both found only on oak seedlings under oak canopy. This pattern is predicted by the theory that mid-seral EM species will be adapted for dispersal--allowing them to quickly establish on pine seedlings under oak canopy--while late-seral EM species show limited dispersal (Jumpponen et al. 2002, Ishida et al. 2008). Seedlings can acquire fungi from either seral group via association with mature fungal individuals, but only mid-seral fungi can infect seedlings via spore dispersal and germination of new fungal individuals.

In contrast to the fungi above, *Tuber* sp. 4 was found exclusively on pine seedlings planted under oak. This species may represent a seedling-adapted life strategy, in which it takes advantage of the pine seedlings' difficulty in finding suitable mycorrhizal associates under oak canopy. *Russula* sp. 5 was likewise found exclusively on oak seedlings under pine canopy, so oak seedlings may also be more available to associate with newly-germinated fungi--as opposed to mature, canopy-fed CMNs--when planted heterospecifically.

This study provides first evidence of a late-seral canopy tree species suppressing seedling recruitment of a mid-seral species by affecting the community composition of EM fungi available in the soil for seedlings to associate with. Late-seral fungal communities were found to be both more diverse and more compositionally consistent

than mid-seral fungal communities, suggesting that EM fungi can converge on a particular late-seral community via multiple successional trajectories. Further studies are required to determine what, if any, role soil properties play in the succession of EM fungi.

Table 1. EM fungi with preferences for pine or oak. Species with a Fisher's exact test p-value of <0.1 for either canopy type or seedling identity are included. The frequency with which each species was found under oak canopy and pine canopy as well as on oak seedlings and pine seedlings is listed along with the p-values for both tests.

Taxon	Abundances				P-value of Fisher's exact test	
	Oak canopy	Pine canopy	Oak seedlings	Pine seedlings	canopy type	seedling sp.
<i>Amanita flavoconia</i>	0	4	3	1	0.052	0.276
<i>Cenococcum geophilum</i>	27	13	22	18	0.017	0.188
<i>Clavulina sp. 1</i>	0	4	3	1	0.052	0.276
<i>Cortinarius lividoochraceus</i>	4	0	4	0	0.069	0.050
<i>Cortinarius sp. 3</i>	5	0	4	1	0.035	0.156
<i>Lactarius sp. 1</i>	4	0	2	2	0.069	0.653
<i>Lactarius sp. 2</i>	1	3	0	4	0.284	0.073
<i>Pachyphloeus sp. 1</i>	0	10	4	6	0.001	0.434
<i>Peziza sp. 1</i>	1	11	7	5	0.002	0.319
<i>Peziza sp. 2</i>	0	4	2	2	0.052	0.653
<i>Pezizaceae sp 5</i>	0	7	3	4	0.005	0.552
<i>Piloderma sp. 1</i>	9	0	3	6	0.002	0.297
<i>Rhizopogon fuscorubens</i>	3	1	0	4	0.337	0.073
<i>Russula lutea</i>	4	0	3	1	0.069	0.276
<i>Russula raoultii</i>	5	0	1	4	0.035	0.215
<i>Russula sp. 5</i>	0	4	4	0	0.052	0.050
<i>Russula sp. 7</i>	9	0	9	0	0.002	0.001
<i>Russula sp. 8</i>	4	0	3	1	0.069	0.276
<i>Russula sp. 9</i>	0	6	1	5	0.011	0.129
<i>Sebacina sp. 1</i>	7	1	2	6	0.040	0.172
<i>Sebacina sp. 2</i>	9	1	3	7	0.012	0.206
<i>Suillus variegatus</i>	1	4	0	5	0.163	0.037

<i>Thelephoraceae</i> sp. 11	3	1	0	4	0.337	0.073
<i>Thelephoraceae</i> sp. 3	0	7	4	3	0.005	0.448
<i>Thelephoraceae</i> sp. 6	0	7	4	3	0.005	0.448
<i>Tuber separans</i>	3	12	7	8	0.009	0.577
<i>Tuber</i> sp. 3	0	11	5	6	<0.001	0.565
<i>Tuber</i> sp. 4	5	0	0	5	0.035	0.037
<i>Tylospora</i> sp. 1	2	5	0	7	0.194	0.010

Table 2. Results of soil analyses for ten plots.

Plot	Canopy	% water by weight		pH		% N		C:N		% Sand	% Clay
		mineral	organic	mineral	organic	mineral	organic	mineral	organic	mineral	mineral
11O	Oak	18.4	46.4	3.75	4.91	0.076	0.983	22.0	22.9	33.0	16.7
11P	Pine	21.2	44.4	4.22	4.31	0.136	0.655	17.4	28.7	34.9	12.9
24O	Oak	21.5	50.6	3.95	5.00	0.146	1.163	20.4	29.6	27.7	25.0
24P	Pine	25.4	52.6	5.32	5.58	0.200	0.959	14.4	27.7	32.6	18.9
7O	Oak	20.0	46.3	3.36	4.44	0.141	1.356	35.1	24.9	24.8	25.0
7P	Pine	18.8	38.0	4.30	5.10	0.097	0.684	20.5	28.9	30.8	7.3
CfO	Oak	17.4	44.3	3.60	4.99	0.085	0.940	26.9	28.6	42.0	17.4
CfP	Pine	18.3	37.8	4.57	4.67	0.141	0.793	18.2	29.8	50.8	6.5
CnO	Oak	17.6	41.3	3.44	5.49	0.103	1.177	27.7	23.6	24.7	24.6
CnP	Pine	19.4	54.8	3.96	3.96	0.190	0.888	22.7	33.2	52.4	7.5

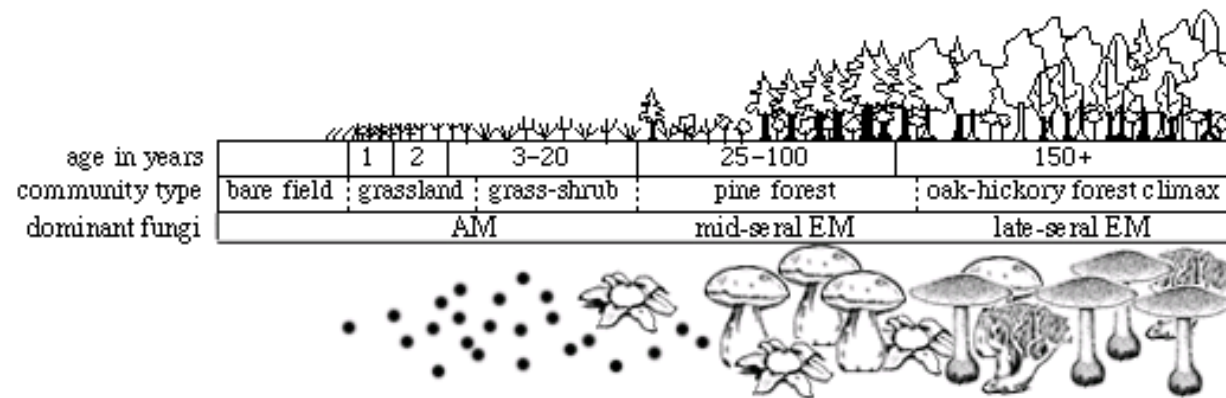


Figure 1. Old-field succession of mycorrhizal fungi (modified from Odum 1971).

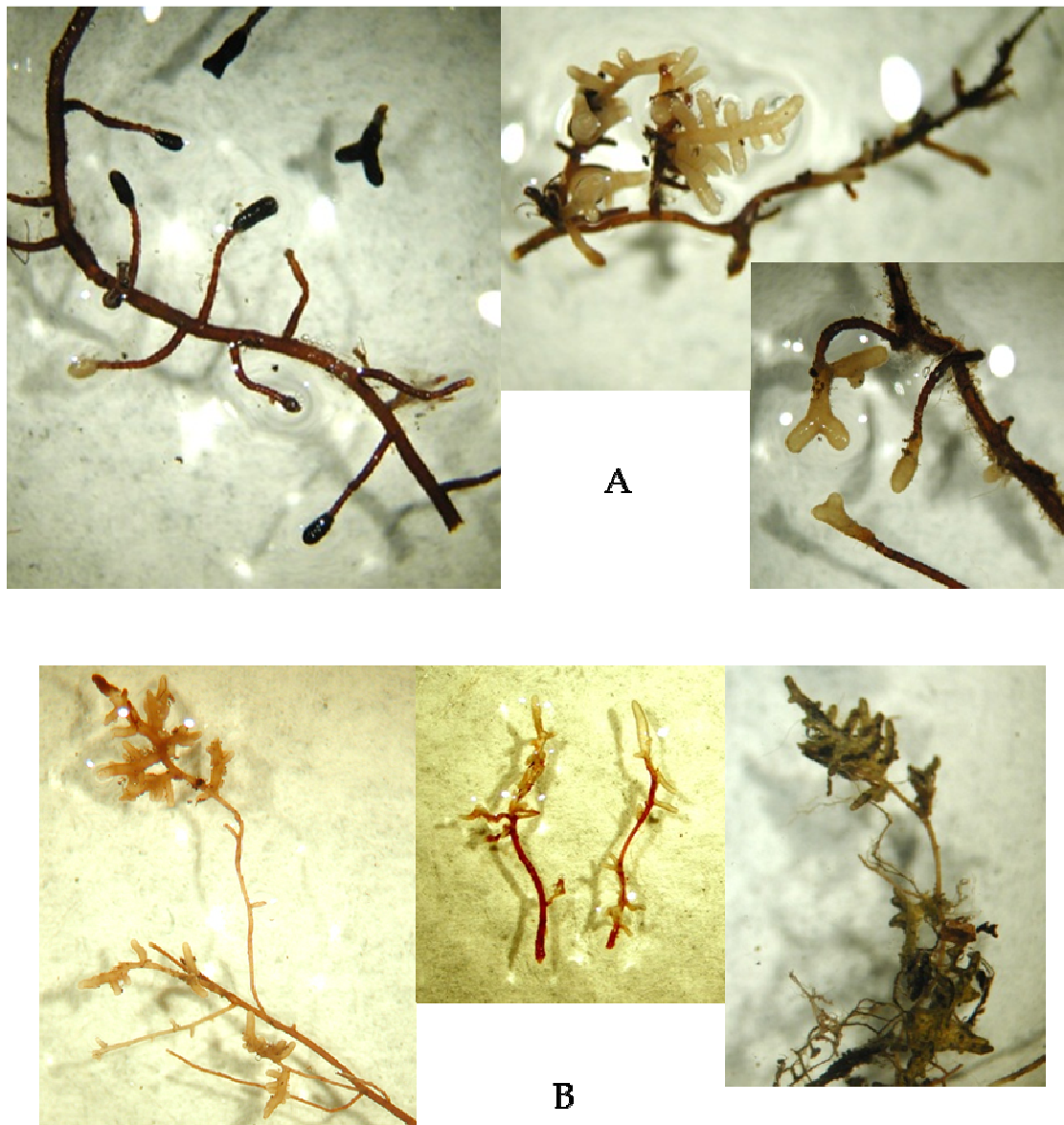


Figure 2. Examples of EM root tip morphotypes and comparison of root morphologies. Pine roots (A) are thicker and less profusely branched than oak roots (B), and tend to have larger EM root tips. Typical pine mycorrhizae include Pezizales (right), Russulaceae (center), and *Cenococcum* (left). Exceptionally large oak mycorrhizae are shown here, including Thelephoraceae (right), Boletales (center), and unknown (left).

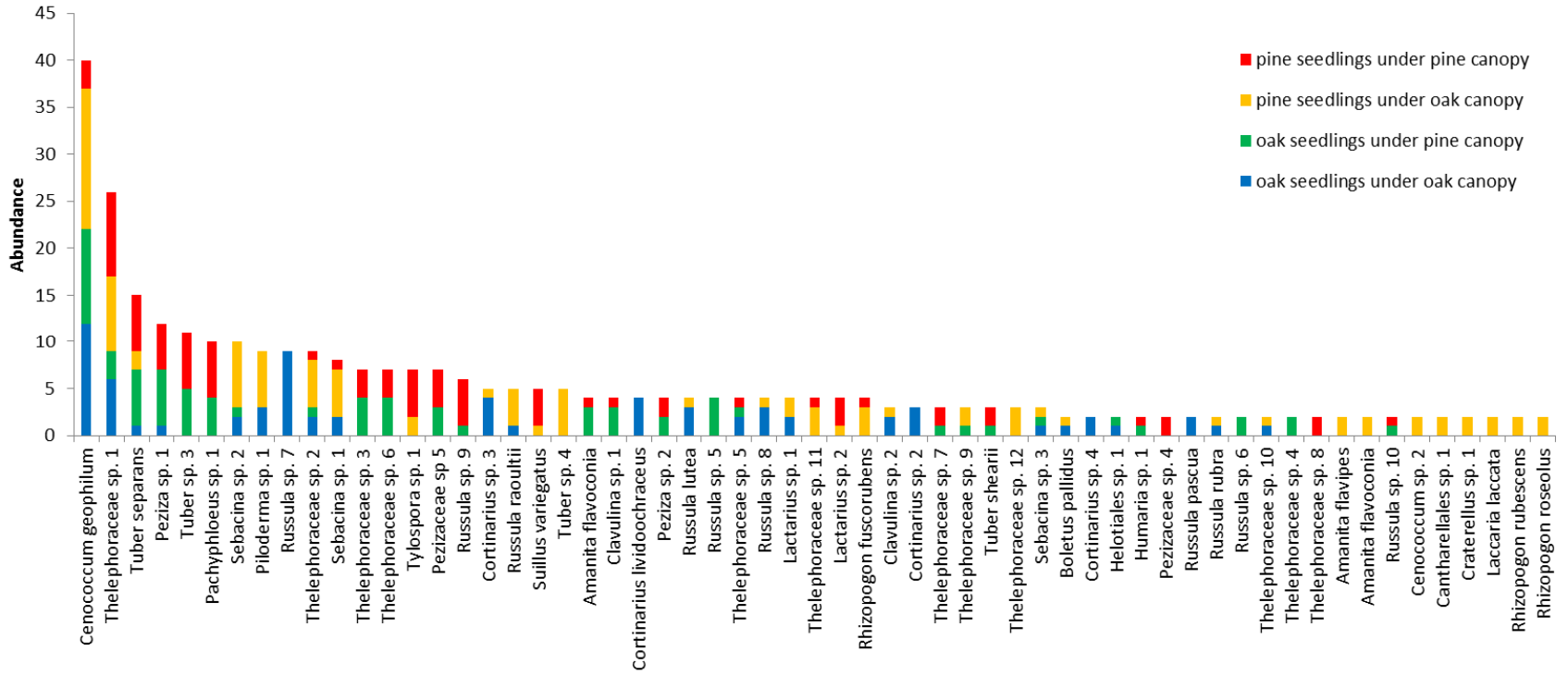


Figure 3. Abundances of 60 OTUs across four treatment types. Singletons not shown.

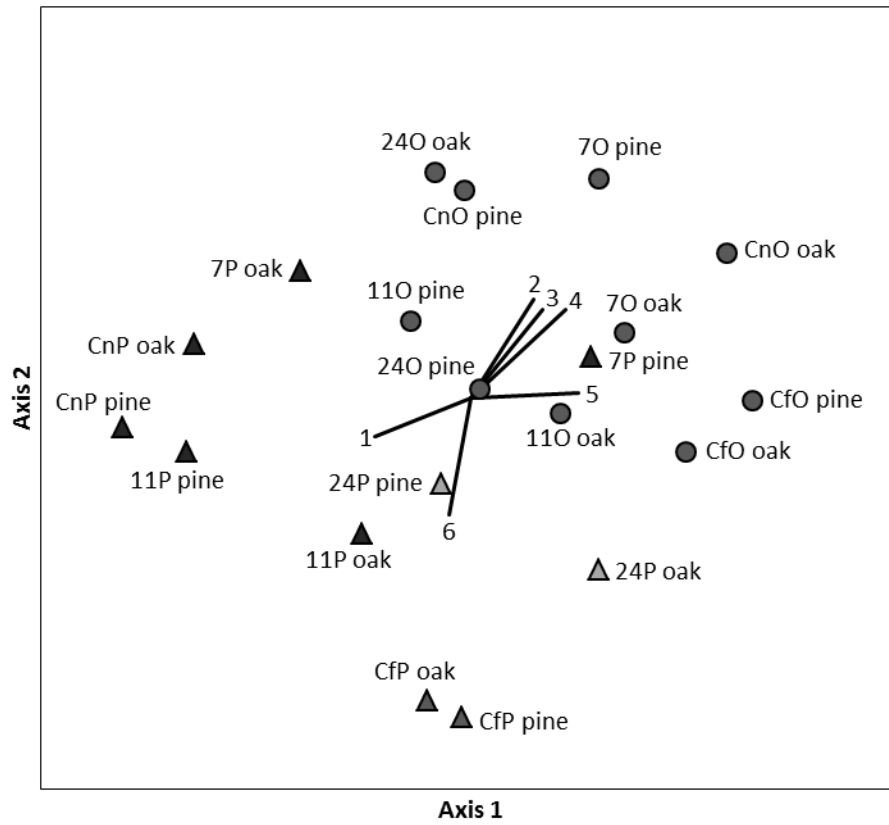


Figure 4. NMS ordination of plots in EM species space, showing Axis 1 (11%) vs Axis 2 (21%). Labels are plot IDs followed by seedling species ("pine" or "oak"). Circles represent oak-canopy plots, which form a single, robust cluster. Pine-canopy plots are marked with triangles and shaded according to cluster analysis groups. Vectors indicate post-hoc correlations of soil variables: 1) organic horizon C:N, 2) mineral horizon C:N, 3) organic horizon N, 4) mineral horizon clay, 5) organic horizon pH, 6) mineral horizon pH.

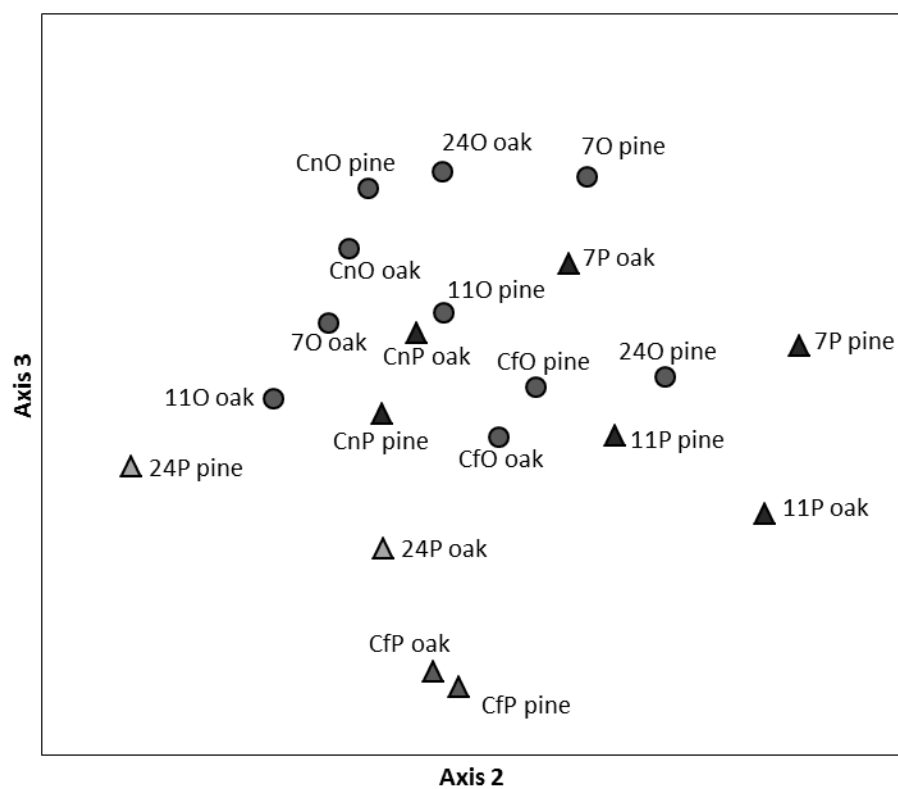


Figure 5. NMS ordination of plots in EM species space, showing Axis 2 vs Axis 3 (23%).

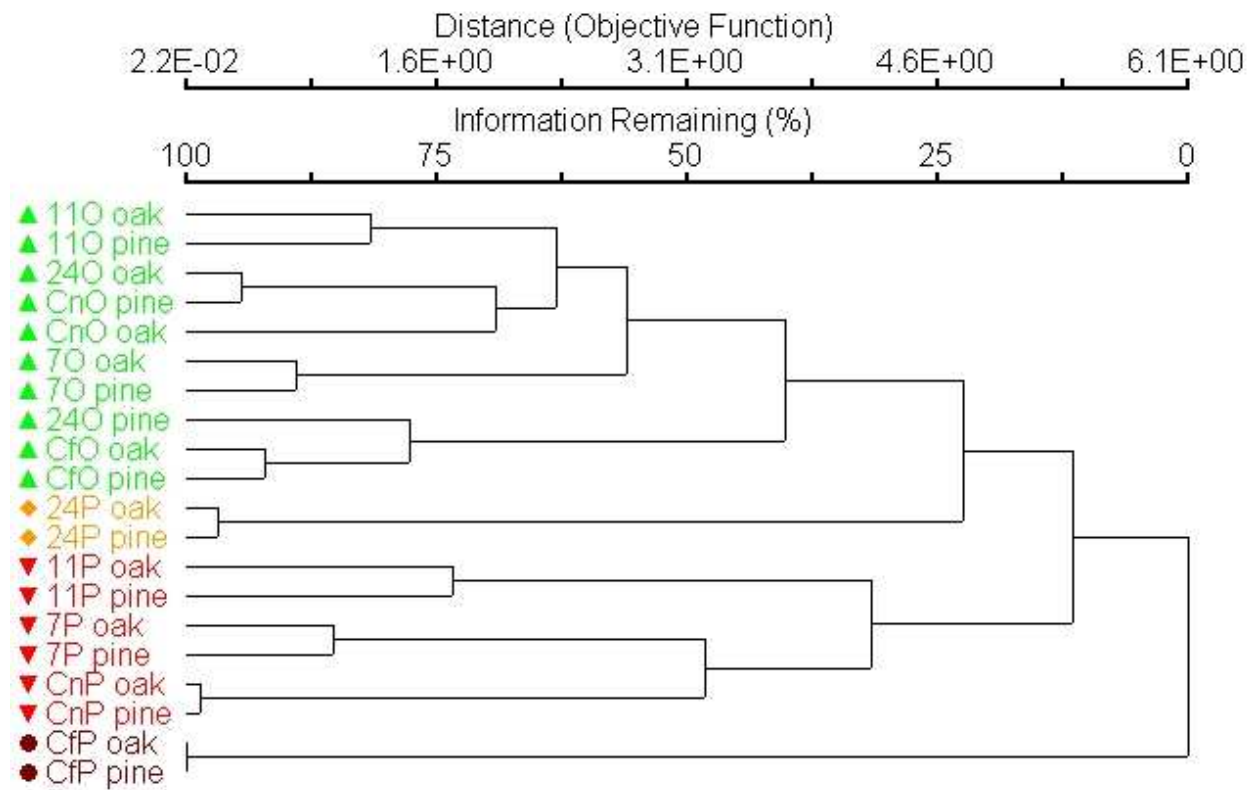


Figure 6. Cluster analysis dendrogram of EM community composition across plots. Oak-canopy and pine-canopy plots cluster separately, and oak plots have a more consistent composition than do pine plots.

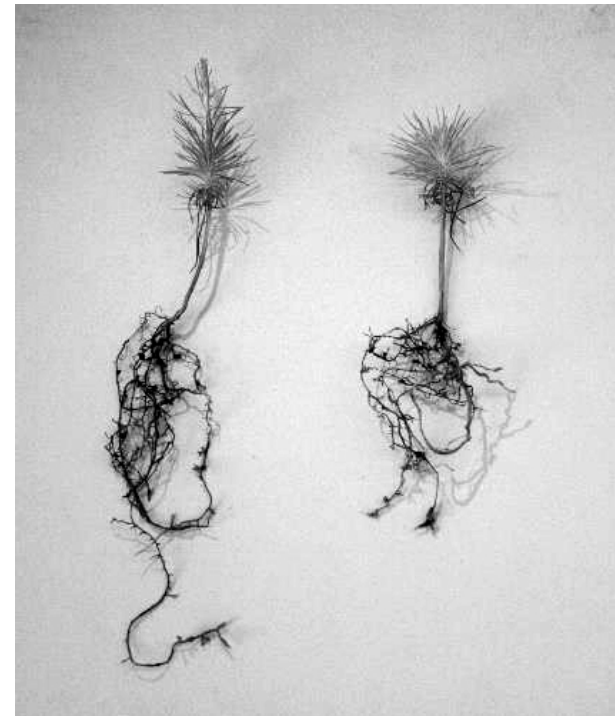
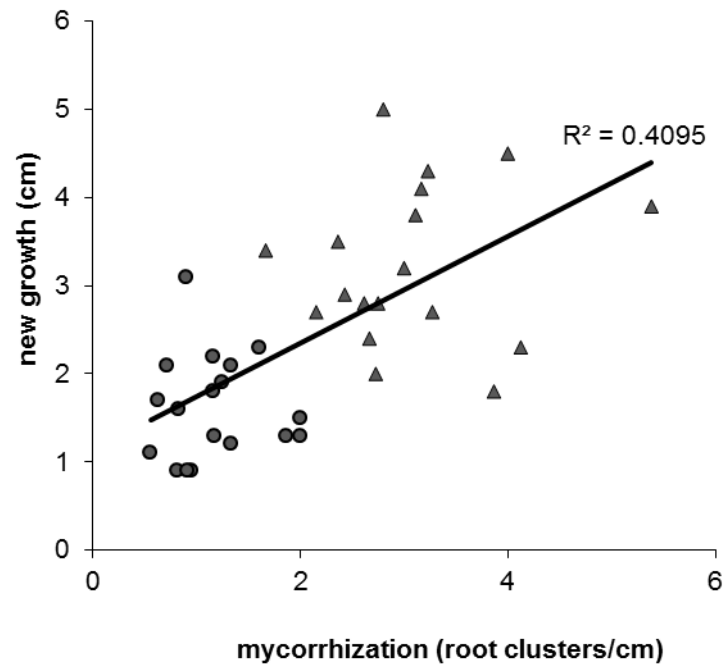


Figure 7 (a) New shoot growth plotted against root mycorrhization of pine seedlings. Circles represent seedlings planted under oak canopy, and triangles represent seedlings planted under pine canopy. **(b)** Visual comparison of pine seedlings planted under pine canopy (left) and under oak canopy (right) at the same site.

2. Seedling identity drives ectomycorrhizal community assembly in the absence of canopy tree influence

2.1 Introduction

The classic model of old-field succession describes changes in plant community composition, but the soil microbiome undergoes a succession of its own. Where the above- and below-ground communities interact, there is the potential for plants and fungi to influence each other's successional trajectories. The mutualism between trees and mycorrhizal fungi is one of the clearest and most widespread examples of this reciprocal influence. Mycorrhizal succession has been documented in a number of study systems, such as glacial forefronts (Cazares et al. 2005), recent volcanic deposits (Ishida et al. 2008), riparian zones (Piotrowski et al. 2008), and sites recovering from clear-cut logging (Horton et al. 2005). Establishing causality, however, has proved difficult -- some studies point to mycorrhizal fungi as drivers of plant diversity (van der Heijden et al. 1998), while others suggest that host plants drive fungal diversity (Jumpponen et al. 2002).

Ectomycorrhizal (EM) fungi are a highly diverse group composed of many lineages independently converging on the same life strategy. One component of their functional diversity is their preference for associating with particular host taxa. While relatively few EM fungi are strict specialists, most express some degree of host preference (Smith et al. 2009), which means that EM community composition is

influenced by the availability of different hosts. Additionally, an EM fungal species can provide better services to one host taxon than to another, even when it freely associates with both hosts (Pande et al. 2007), such that the availability of different EM fungi can, in turn, affect plant community composition.

To further complicate these interactions, edaphic properties have the potential to influence -- or be altered by -- community composition of both plants and fungi.

Nutrient availability, in particular, plays a key role in the determination of EM fungal diversity and is one potential cause of the seral shift in EM community composition. EM species richness increases asymptotically with soil fertility, and peak species richness coincides with high heterogeneity, as well as amount, of available nitrogen (Kranabetter et al. 2009). Functional diversity of EM traits, such as exploration type, correlates with increased soil fertility as well as the heterogeneity of available microsites (Tedersoo et al. 2008, Kranabetter et al. 2009). Soil chemistry appears to be the primary factor affecting the re-establishment of EM fungi in disturbed areas (Jones et al. 2003).

It remains unclear whether plant community composition is responsible for controlling these properties or not. For instance, anthropogenically altered edaphic conditions have been implicated in the decline of oak forests (Orwig and Abrams 1994, Dey et al. 2008), but changes in soil properties may be an effect, rather than a cause, of increased pine dominance (García-Barrios and González-Espinosa 2004). Plant traits such as leaf structure can drive litter and soil properties, including rate of decay

(Cornelissen et al. 1999). Thus, there is the potential not only for direct interactions between fungal and plant communities, but also for indirect effects via the soil.

The North Carolina Piedmont region provides an ideal system in which to study seral changes in EM community composition. The Duke Forest, in particular, inspired one of the first theoretical models of succession, wherein abandoned fields become colonized first with shrubs and forbs and then with pines, which are in turn replaced by oak-hickory forest (Oosting 1942). Previously, a reciprocal transplant of white oak (*Quercus alba*) and loblolly pine (*Pinus taeda*) seedlings was conducted in paired plots of oak-dominated and pine-dominated stands within Duke Forest. This study suggested that the fungal associations of adult trees determine which EM species are dominant in the soil and therefore drive the availability of EM fungi for seedlings to associate with (Chapter 1). Contrary to many studies, I did not recover a strong signal of edaphic conditions affecting EM community composition. However, the correlation between canopy type and edaphics makes it difficult to establish a direct causal link between canopy species and EM fungi on seedlings.

Here, I investigate the degree to which adult trees influence the availability of EM fungi for seedlings by comparing bioassays in the field with bioassays conducted in the laboratory without the influence of adult trees. Based on the results from field plots, I hypothesize that EM communities on pine and oak seedlings will converge in the absence of adults, and that soil properties will have a negligible effect on most EM

species. I also predict that some EM species will show a distinct preference for pine or oak seedlings, and that this signal will be much more prominent without canopy influence.

2.2 Methods

Ten paired field plots were selected for a previous study, with each pair consisting of one plot dominated by white oak and the other dominated by loblolly pine. Soil was collected from these plots -- including the organic layer and top ~5cm of the A-horizon, but excluding litter -- and stored in paper bags. These soils were allowed to air dry at room temperature for a minimum of 3 months. Thoroughly drying the soils kills off any mycelium and leaves only spores as fungal inoculum (Avis and Charvat 2005, Taylor and Bruns 1999). Acorns and pine seeds were surface sterilized, cold-stratified and germinated in sterilized perlite medium.

Ten Cone-tainers (RLC-4 Pine Cell, 2.5×16 cm; Stuewe and Sons) per plot were filled with dried field soil, and seedlings were transplanted into each and immediately watered. The Cone-tainers were split evenly between bioassay treatments: two oak seedlings or two pine seedlings in each. A thin layer of sterilized sand was added on top to prevent cross-contamination when the plants were watered. Cone-tainers were kept in a growth chamber at 24 °C and 14 hours of simulated daylight, and were watered every other day.

The seedlings grew undisturbed for 6 months to allow time for EM fungal spores to germinate and infect roots, then were harvested for their mycorrhized root-tips. The roots were carefully separated from the soil and examined under a dissecting microscope, and 2-3 tips of each morphotype were sampled per seedling. Each root tip was placed in 20µl extraction solution and DNA was extracted following the Extract-N-Amp (Sigma-Aldrich, Inc.) protocol. For use as a DNA barcode, the internal transcribed spacer region (ITS) of ribosomal DNA was amplified with the forward primer ITS-1F (Gardes and Bruns 1993) and the reverse primer ITS4 (White et al. 1990), and then capillary sequenced using Big Dye 3.1 on a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA). EM fungi from mycorrhized root tips were then identified by comparison against GenBank as well as against the operational taxonomic units (OTUs) delineated in the previous field study. Sequences will be made available via submission to GenBank.

At the time of harvest, a small soil sample from each Cone-tainer was saved and frozen to preserve DNA. When harvesting was complete, the five soil samples per treatment were pooled and subsampled, and fresh soil samples were obtained from field plots for comparison. DNA was then extracted with a PowerSoil Extraction Kit (Mo Bio), and the large subunit (LSU) region of ribosomal DNA was amplified with tagged primers. These amplicons were pooled then sequenced on a Roche 454 GS FLX (454 Life Sciences).

The high-throughput LSU data were processed in QIIME version 1.8 (Caporaso et al. 2010) using the `denoise_wrapper.py` function (Reeder and Knight 2010) for denoising and the `pick_otus.py` function (Edgar 2010) for assigning species-level delineations. The resulting OTU table was imported into PC-Ord (McCune and Mefford 1999) and abundances were relativized by species to reduce the influence of the most abundant species and mitigate any phylogenetic biases that may have arisen during amplification or sequencing. Hierarchical clustering was accomplished with the flexible-beta joining algorithm (Lance and Williams 1966) set to a beta value of -0.25, which approximates Ward's Method (McCune and Grace 2002). For non-metric multidimensional scaling (NMS), a two-dimensional solution was selected using a step-down procedure. Vectors were then overlaid for soil properties that correlate with community composition.

2.3 Results

Direct sequencing of EM root tips recovered 11 species on oak seedlings and 8 on pine seedlings, with individual seedlings most commonly hosting 2-4 species. Of these 19 EM species, only 12 were found more than once during this study (Fig. 8). No differences in shoot growth or root mycorrhization were observed between soil treatments.

High-throughput soil sequencing detected a total of 1440 OTUs, of which only 273 appeared in multiple treatments and/or multiple plots and thus could be informative for community analysis. I retained 95 OTUs from field soils and 192 OTUS from growth chamber soils. Fungal communities in field soils cluster according to canopy type (Fig. 9), which is consistent with previously collected root-tip data. In growth chamber soils, however, most fungal communities cluster according to the identity of the seedlings growing in the soil (Fig. 10).

This grouping pattern among soil fungal communities can also be seen in the NMS ordinations (Figs. 11, 12). With two-dimensional solutions, NMS can account for 68.3% of the variance in field soils and 60.6% of the variance in growth chamber soils. In the field, several soil properties -- including nitrogen and C:N ratio of both the mineral and organic horizons -- correlate with fungal community composition. But in the growth chamber, these same soil properties for the most part failed to correlate with community composition, mineral horizon pH being the only exception.

2.4 Discussion

The same two EM species found in greatest abundance on seedling root tips in the field also dominated in this study. These species -- *Cenococcum geophilum* and Thelephoraceae sp. 1 -- appear to be generalists with regard to host identity. Previous studies have observed lower relative abundances of *C. geophilum* in bioassay pots than in

the field, and it has been suggested that *C. geophilum* is a poor competitor when confined in close proximity to other species (Avis and Charvat 2005, Dickie et al. 2002). In this study system, however, *C. geophilum* almost always co-occurred with other species on the same seedling, and I observed no difference in its extent of colonization between field and growth chamber studies. Indeed, *C. geophilum* was the first species to form visible EM root tips on both oak and pine seedlings in the growth chamber (pers. obs.), and it remained persistently common as other species germinated and formed mycorrhizae.

Some species, such as *Sebacina* sp. 1 and Thelephoraceae sp. 2, which previously showed a preference for canopy type are now expressing a preference for seedling identity instead. This change suggests that canopy trees exert a direct influence on the abundance of certain EM taxa, rather than an indirect effect via soil conditions. The genus *Rhizopogon* showed a dramatic increase in its abundance when moved from the field to laboratory conditions, which supports its status as a colonization-adapted taxon (Peay and Bruns 2009) that readily colonizes root tips from newly germinated spores.

Field soils recovered the same pattern of segregating by canopy type as previously found on seedlings in the field -- an interesting result given that all fungi were amplified for sequencing, not just EM fungi. One possible explanation is that EM fungi are so dominant in the upper soil horizons that non-EM fungi have a negligible effect on the community analyses; however, this seems unlikely given that EM

mycelium accounts for only a third of the fungal biomass in soil (Hogberg and Hogberg 2002) and that saprophytic fungi predominate in the litter layer (O'Brien et al. 2005).

Assuming there are significant quantities of non-EM fungi present, they could be directly responding to either soil properties or canopy type. This would generate differences in non-EM diversity between oak- and pine-dominated plots, such that non-EM and EM communities have a correlative but not causative relationship.

In growth chamber soils, the fungal communities clustered by seedling identity regardless of where the soil originated, demonstrating that the adult trees' preference for certain EM fungi was, indeed, the primary driver of EM diversity found on seedlings in the field. I not only saw an absence of grouping according to canopy type, but also a much stronger signal of seedling identity effects, which were present in the field study but partially obscured by canopy effects. Only one treatment -- pine seedlings planted in soil from oak plot 11 ("11O pine") -- failed to cluster with conspecific seedlings, appearing within the oak seedling cluster group instead. Interestingly, this does not appear to be a lingering effect of soil origin, since the oak seedlings planted in the same soil ("11O oak") do not cluster especially close to the 11O pine seedlings.

With the influence of canopy trees removed, one soil property -- mineral soil pH -- still correlates with fungal community composition. This vector lies perpendicular to the direction which would indicate a correlation with seedling identity, indicating that pH is not responsible for the differences in fungal communities associated with pine and

oak seedlings. Within the range of possible community compositions preferred by a particular seedling species, variation in pH of the A-horizon does appear to influence which fungi become dominant. But in this system, soil is a secondary factor, and the signal of its influence on fungal communities can easily be overwhelmed by the effect of canopy tree fungal preference.

Again, it is interesting to note that these well-differentiated fungal communities contain both EM and non-EM species. If these soil samples contained a significant diversity of non-EM fungi, then I could conclude that the non-EM community is responding to host identity rather than soil properties. It could also be that canopy trees exert an indirect influence on the non-EM community by favoring certain EM fungi over others. For this to be true, the EM community would have to be a primary driver of non-EM fungal composition. Then, in the absence of canopy trees, seedlings could indirectly affect non-EM fungi by influencing EM fungi.

In conclusion, this study did recover evidence of fungal communities responding to all three direct influences -- canopy type, seedling identity, and soil properties -- but in descending order of importance. The influence of canopy trees on the fungal community is strong enough to partially mask the fungal preferences of the seedlings themselves. Both soil properties and fungi respond to canopy type in the field; this produces the appearance of soil effecting the fungal community, when in fact the relationship is not causative. The influence of canopy type obscures the true effect of A-

horizon pH on fungal community composition, such that it can only be definitively observed in the absence of adult trees. While soil properties have often been implicated as drivers of fungal communities, this study shows that we must be cautious when inferring causal relationships in complex systems.

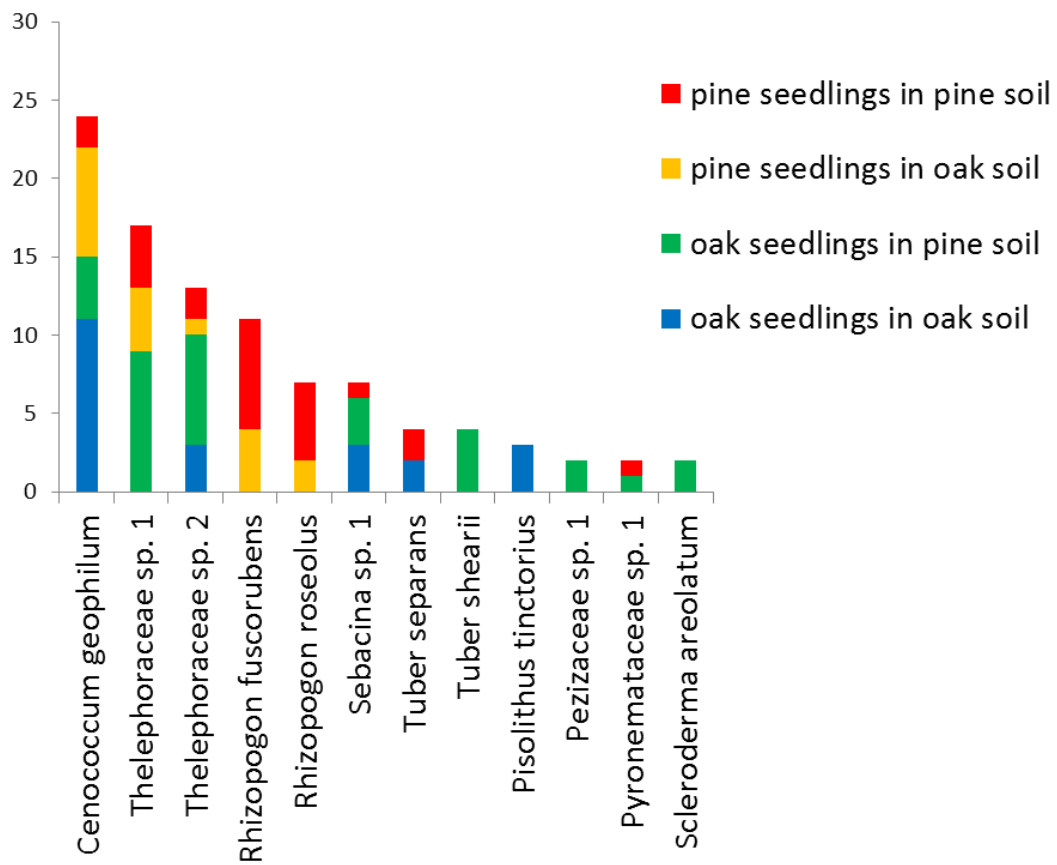


Figure 8. Ranked abundances of EM fungal species found on root tips.

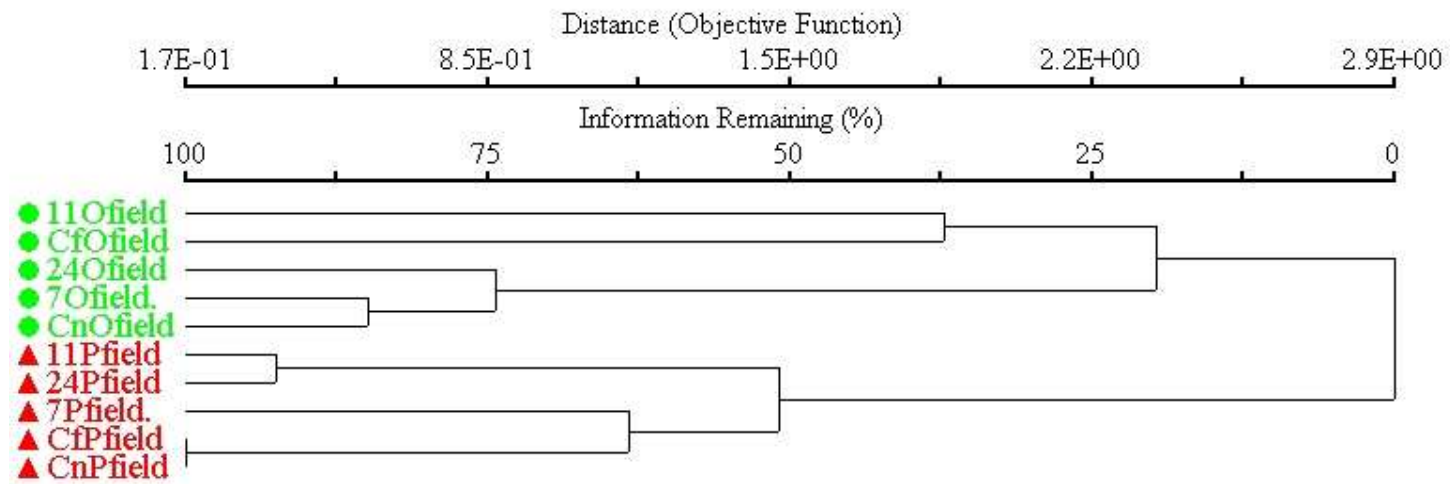


Figure 9. Cluster analysis dendrogram of community composition of soil fungi across field plots. Oak-canopy and pine-canopy plots cluster separately.

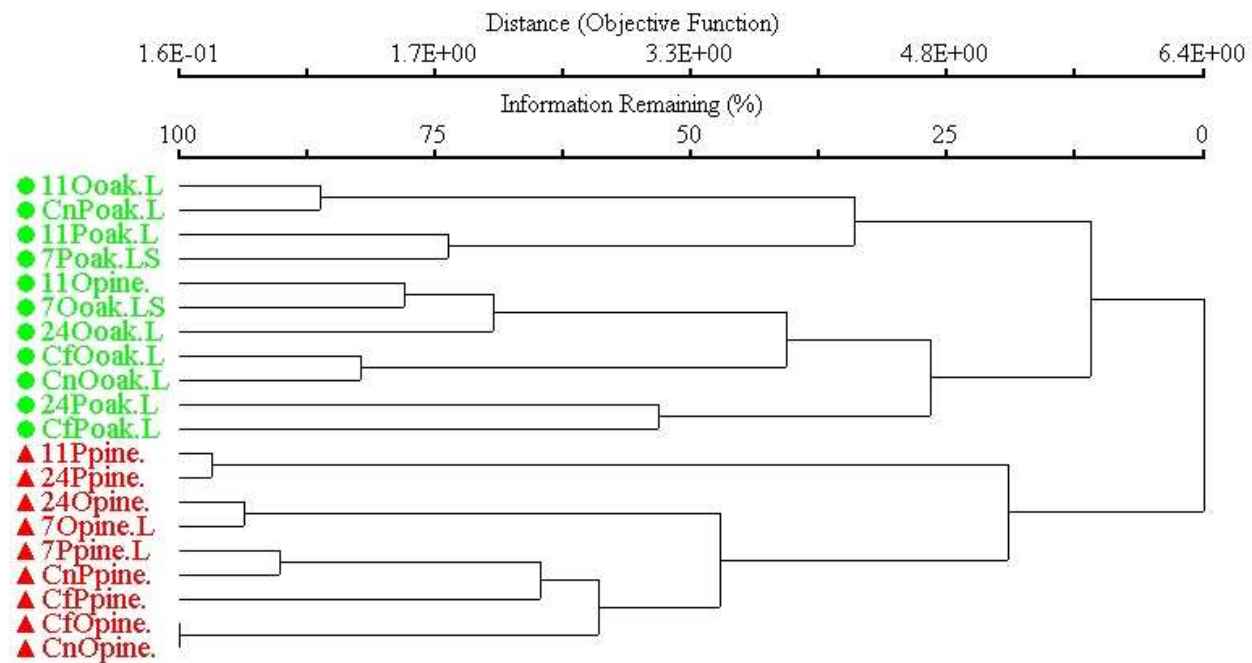


Figure 10. Cluster analysis dendrogram of fungal communities in growth chamber soils. Seedling identity determines cluster group with only one exception (11O pine).

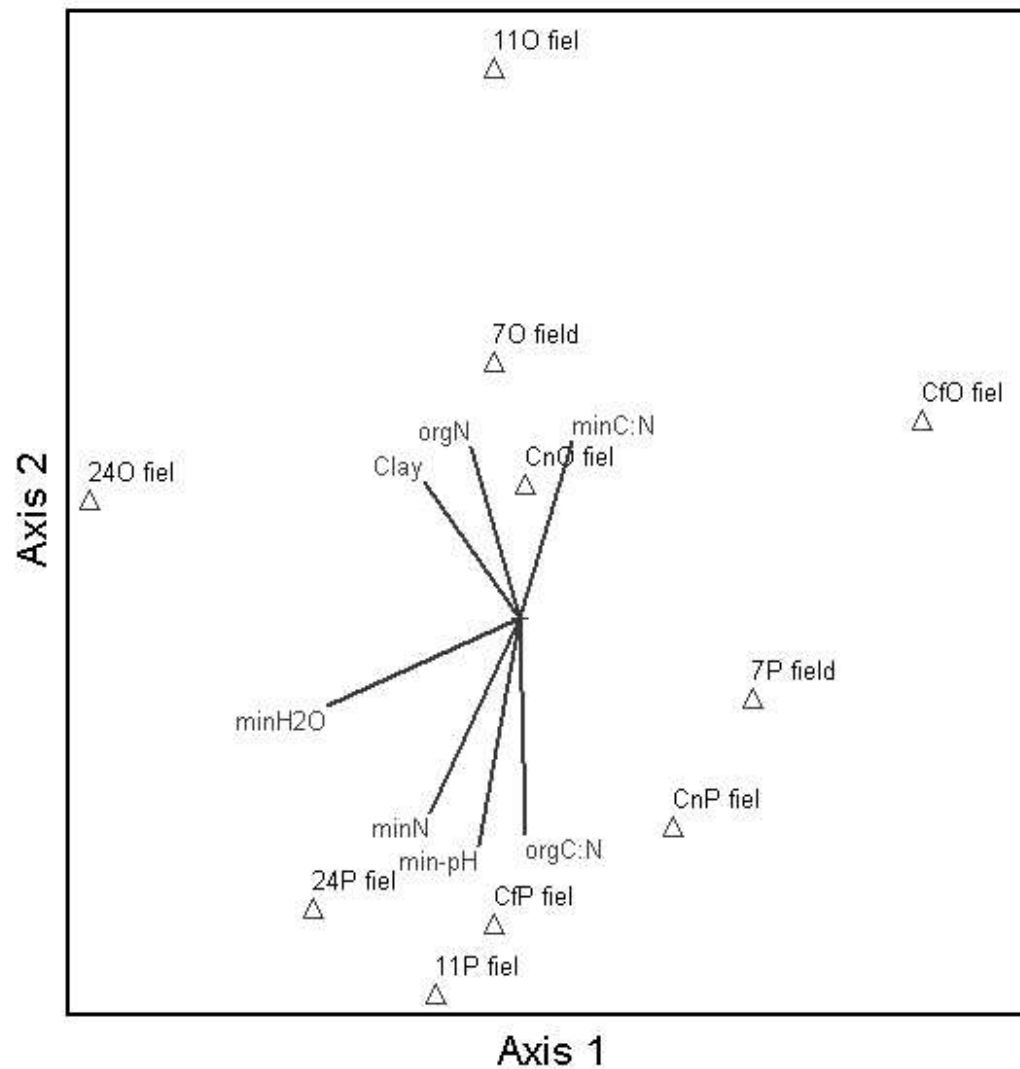


Figure 11. Two-dimensional NMS ordination of field soils in species space. Labels are plot IDs, with "O" for oak canopy and "P" for pine canopy. Vectors indicate post-hoc correlations of soil variables: organic horizon C:N, mineral horizon C:N, organic horizon N, mineral horizon clay, organic horizon pH, mineral horizon pH.

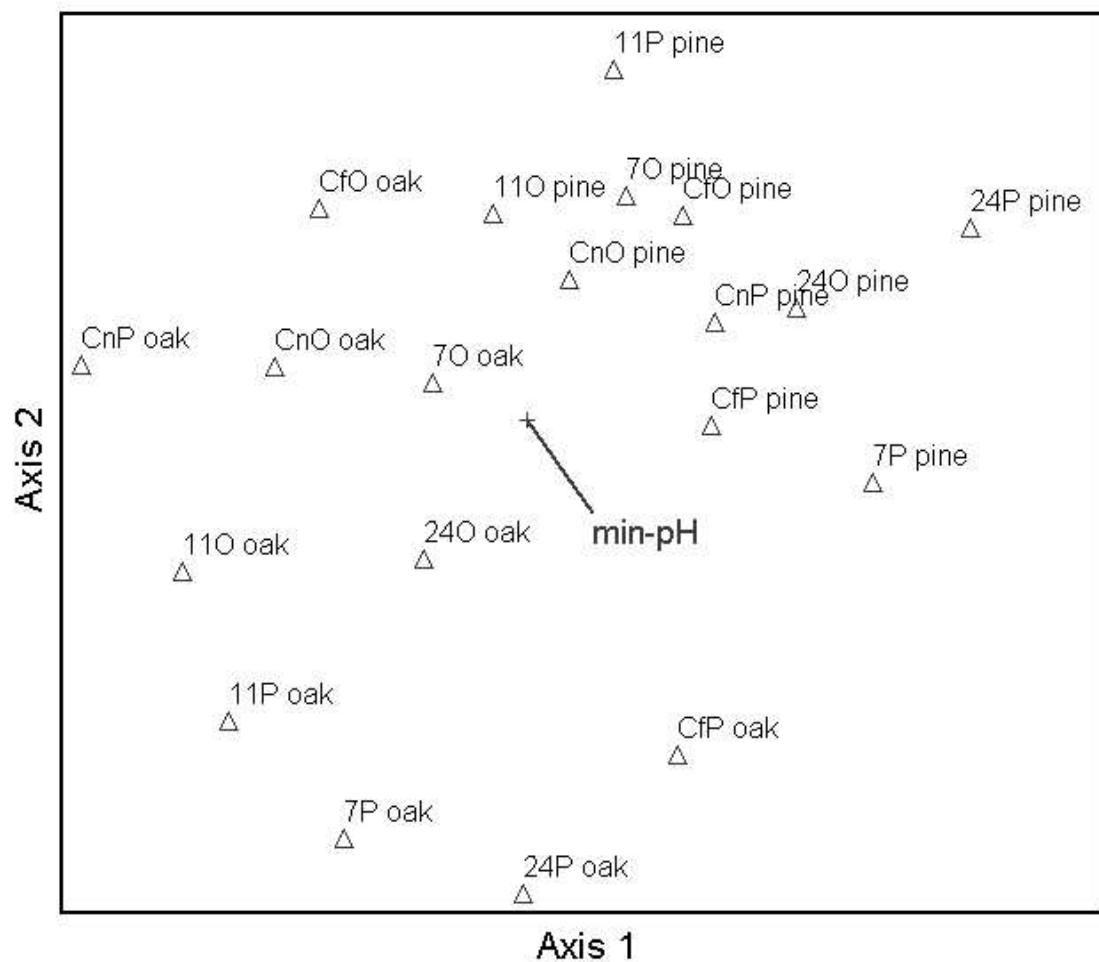


Figure 12. Two-dimensional NMS ordination of growth chamber soils in species space. Labels are plot IDs followed by seedling species ("pine" or "oak"). Without the influence of canopy trees on soil fungi, only one soil property correlates with fungal community composition.

3. Ectomycorrhizal diversity of oak forests in the Sierra Madre Oriental, Mexico

3.1 Introduction

Ectomycorrhizal (EM) fungi play an essential role in the ecology of North American forests. In the EM mutualism, fungal species--primarily from the Agaricomycotina and Pezizomycotina--associate with the roots of dominant tree families such as *Pinaceae*, *Fagaceae*, and *Betulaceae*. The host tree provides the fungus with photosynthates in exchange for nutrients absorbed from the soil, especially nitrogen, though the fungus may also confer secondary benefits such as protection against root pathogens and increased drought tolerance (Smith and Read 2008). This mutualism shows a surprising degree of convergence, having evolved independently in as many as 82 lineages of fungi (Tedersoo and Smith 2013, Tedersoo et al. 2010).

As well as being phylogenetically diverse, EM fungi show a range of life strategies (Lilleskov and Bruns 2003) and hyphal morphology (Agerer 2001), and this functional diversity affects host benefit. Different fungal species vary, for instance, in the quantity of nitrogen they provide to their host (Jones et al. 2009). Generalist EM fungi that freely associate with a wide range of host taxa can confer unequal benefits to different tree species, promoting the growth of one species over another (Pande et al. 2007). Additionally, some degree of host preference is widely prevalent in EM-dominated ecosystems (Smith et al. 2009), and this preference for particular host taxa

may play an important role in plant community assembly (Tedersoo et al. 2008). However, the most commonly fruiting EM species often do not correspond to the species that dominate on the roots of trees (Horton and Bruns 2001), so a survey of fruit bodies alone may not yield accurate information about the relative ecological importance of different species.

Montane forests of Mexico and Central America are of particular interest to fungal systematists because they show a biogeographic trend of EM fungi migrating southward from temperate North America along with their host trees (Halling 2001). The dry scrubland surrounding the Sierra Madre Oriental (SMO) is dominated by plant taxa such as *Yucca*, *Cordia*, and *Prosopis* which associate with arbuscular mycorrhizal (AM) fungi, so the EM-dominated montane forests may act as biogeographic "sky islands" in a sea of AM-dominated lowlands. Subtropical and tropical oak forests host a wealth of unique ectomycorrhizal species (Halling and Mueller 2002) due to a combination of biogeographic and environmental factors.

In this study, I conducted a survey of below-ground EM diversity in the SMO mountains of northeastern Mexico as a complement to ongoing taxonomic investigations of EM fruit bodies from the region (e.g. Guevara et al. 2013, Healy et al. 2009). These data allow us to address several questions that could not be explored using fruit bodies alone: (1) Do the EM assemblages show evidence of biogeographic isolation? (2) Are these communities structured similarly to other oak-dominated systems? (3) Does the

co-occurrence of pines at high elevation affect community composition on oaks?

3.2 Methods

3.2.1 Study sites

Sampling occurred at eight sites in the vicinities of Monterrey, Nuevo León and Victoria, Tamaulipas in northeastern Mexico during July and August, 2008 (Table 3). Sites ranged in elevation from 700 m to 2700 m and could be characterized as oak-dominated woodland or mixed oak/pine forest, often transitioning into pine-dominated forests at higher elevations. Oak species include *Quercus polymorpha*, *Q. canbyi*, *Q. laeta*, and *Q. affinis*, among others, though an individual site was often dominated by only one or two oak species. While temperature and precipitation are somewhat variable across the region and generally undocumented, the Balcón de Montezuma station (23° 36' N, 99° 12' W, 1284m) records a mean annual temperature of 17.8° C and annual rainfall of 1150 mm.

3.2.2 EM root sampling and molecular identification

Oak EM root tips were collected from the A-horizon at depths <20 cm. At each site, roots were sampled from 7-11 trees, for a total of 70 trees across both states. Eight to ten EM tips were selected from each root, attempting to sample the full range of morphological diversity. These root tips were washed in 70% ethanol and stored in cetyl trimethylammonium bromide (CTAB) at room temperature until processed.

DNA was extracted according to the protocol of Zolan and Pukkila (1986). The internal transcribed spacer region (ITS) of nuclear ribosomal DNA was amplified by polymerase chain reaction using either the universal forward primer ITS1 (White et al. 1990) or the fungal-specific forward primer ITS1-F (Gardes and Bruns 1993) and the reverse primer ITS4 (White et al. 1990). Capillary sequencing of ITS fragments was conducted using Big Dye v3.1 and visualized on a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA).

Sequences were manually edited and assembled at 100% similarity in Sequencher 4.5 (Gene Codes, Ann Arbor, MI) to check for duplicate haplotypes. If a given haplotype was sequenced multiple times from the same root sample, these root tips were assumed to be pseudoreplicates of an individual fungus, and the ITS sequence was recorded only once. All sequences were then assembled at 97% similarity to assign them to organizational taxonomic units (OTUs), approximating species-level delineations. Sequences were also compared against GenBank accessions using the BLASTn algorithm to assign genus- and, when possible, species-level identifications.

3.2.3 Analysis and comparison against other regions

Mycorrhizal datasets from three other studies were compared. Two of these studies were conducted in the Koch Natural Area in the Sierra Nevada foothills of California, which is classified as a Mediterranean climate and is roughly similar to the

low-elevation sites in Monterrey in terms of temperature (annual mean 17.8° C), precipitation (annual mean 710 mm) and elevation (400-600m). Oak woodland predominates, with mycorrhizae sampled from blue oak (*Q. douglasii*), interior live oak (*Q. wislizeni*), and foothill pine (*P. sabiniana*) (Smith et al. 2007, Morris et al. 2008). The third study took place in Huizteco Park, Guerrero, southern Mexico, where host trees experience high elevation (2400 to 2550 m) and precipitation (annual mean 1400 mm), and temperatures similar to northeast Mexico (annual mean 18° C). The vegetation is classified as tropical montane cloud forest, and EM fungi were sampled from the evergreen *Q. laurina* and deciduous *Q. crassifolia* (Morris et al. 2009). Like northeast Mexico, both of these regions have relatively low EM host diversity, and they allow for an examination of geographic distance, moisture regime, and co-occurring host species on EM community composition.

Data from this study were first ordinated at the species level. Because of the high percentage of unique and unidentifiable species, it would be impossible to compare this study to mycorrhizal surveys in other regions without saturating the Bray-Curtis distance metric, so species data were reassigned to genera for the purpose of comparison. This level of phylogenetic grouping also alleviates the problem that one of the two most common genera, *Tomentella*, does not divide into well-differentiated species based on rDNA sequence analysis.

Seven plots and 20 species were retained in the species-level analysis. Only

species appearing at more than one plot were included, and two plots ("mining road" and "Estanzuela high") were removed because they did not have sufficient shared species. For the genus-level analysis, all plots except for the mining road were retained, and 13 shared genera were used. The multi-region ordination involved 8 plots from SMO, 7 plots from Koch, and 4 plots from Huizteco for a total of 19 plots, with 40 genera shared between at least two plots. When using SMO data only, the abundances were relativized by species/genus maximum. Because of differences in sampling quantity between studies, the ordination of multiple studies used abundances relativized by plot instead.

Nonmetric multidimensional scaling (NMS) is an ordination method particularly well-suited for handling species data (McCune and Grace 2002). The optimum number of NMS axes for each analysis was determined using a stepdown procedure. Varimax rotation was then implemented, and vectors indicating correlations with environmental variables were overlaid post-hoc. Cluster analysis was then explored as a method for classifying sites according to their EM assemblages. (Gower 1967). The Bray-Curtis distance measure and flexible beta joining algorithm were implemented, which preserves spatial relationships among samples, so cluster groups could be overlaid on NMS ordinations for interpretation. Mantel's test was used to determine the optimal number of cluster groups. Ten thousand permutations on a one-tailed test were used to calculate a p-value for the significance of this difference. NMS and cluster analyses were

conducted in PC-Ord version 5 (McCune and Mefford 1999). Mantel's test results were produced in R version 2.9.1 (R Development Core Team 2009) using the "ecodist" package (Goslee and Urban 2007).

3.3 Results

Of the approximately 630 root tips sampled, 274 yielded sequences. Seventy-three of these sequences were pseudoreplicates, where a putative individual was found on multiple root tips from the same root sample. This leaves 201 unique sequences, of which 174 could be identified as fungi likely to participate in an EM symbiosis (Table 4). Eighty-one of the 174 EM sequences had a top BLAST hit with percent similarity at or above 97%. However, only 33 of these BLAST searches yielded GenBank accessions with sufficient information to make a species-level identification for the sequence in question. The genera *Tuber* and *Tomentella* were recovered most frequently, with *Sebacina* and *Inocybe* also common. Despite roughly even sampling between sites, more sequences were recovered from sites in Victoria than in Monterrey, possibly because Monterrey was drier at the time of sampling (pers. obs.), so the EM tissue was less fresh. At 97% similarity, these sequences grouped into 44 OTUs from Nuevo Leon, 84 OTUs from Tamaulipas, and 117 OTUs combined.

Because most sequences could not be identified down to the species level, OTU designations were used in lieu of species for the species-based ordination. For both SMO

ordinations, two-dimensional solutions were selected, converging on final stress values of 11.4 for species-based and 6.12 for genus-based ordinations (Fig. 13). The species-based ordination explained 49.4% of the variance on Axis 1 and 32.2% on Axis 2, while the genus-based ordination explained 54.6% on Axis 1 and 32.1% on Axis 2. A three-dimensional solution (Fig. 14) was selected for the multi-region ordination, converging on a final stress of 7.72 with 30.0% of the variance explained on Axis 1, 25.3% on Axis 2, and 37.2% on Axis 3.

Clustering for SMO data yielded high percent chaining -- 14.29% for species-based and 36.36% for genus-based -- though this is likely an artifact of the small number of plots included in the analysis and not a reflection on the quality of the analysis itself. When the plots from other regions were included, the percent chaining fell to 4.67%. While the optimal cluster level of 4 was significant ($p\text{-value}=0.0074$) for the species-based analysis, the Mantel correlation was substantially lower than that of the genus-based analysis ($R_M=0.651$ versus $R_M=0.752$), so it was not represented graphically. The multi-region analysis clustered optimally at 4 groups ($R_M=0.730$, $p\text{-value}=0.0001$), with the 3-group clustering following close behind ($R_M=0.704$, $p\text{-value}=0.0001$), so both were presented for interpretation.

3.4 Discussion

Despite the likelihood of geographic isolation and the low diversity of host trees, EM fungal diversity was high. This study adds to a growing body of literature indicating that host density may be more important than host diversity in the determination of symbiont species richness. Fruit body surveys from montane oak forests in Costa Rica reflect a similar richness of EM fungi supported by even fewer oak species (Halling and Mueller 2005, Mueller et al. 2006). In the lowland neotropics, the high EM diversity found on *Dicymbe corymbosa* -- a tree that forms monodominant stands -- contrasts starkly with the low richness and abundance found on widely-dispersed EM hosts such as the Nyctaginaceae, *Pakaraimaea dipterocarpacea* (Dipterocarpaceae), *Gnetum* (Gnetaceae), and *Coccoloba* (Polygonaceae) (Henkel et al. 2002, Haug et al. 2005, Moyersoen 2006, Setaro et al. 2006, Tedersoo et al. 2010). The positive impact of host density on the diversity of wood-decay, epifoliar, and endophytic fungi has been previously documented (Gilbert et al. 2002, Gilbert et al. 2007, Suryanarayanan et al. 2003), and the species richness of ectomycorrhizal fungi appears to be similarly dependent on host density.

However, two genera -- *Tuber* and *Tomentella* -- predominated in this study, collectively comprising almost 40% of all EM samples. While species richness of EM fungal communities may depend on host density, evenness may reflect some other variable, such as the species richness of host trees. Low evenness, in turn, may affect

ecosystem function, with the dominant EM fungi having a proportionately large influence.

This study shows that a survey based on fruit bodies alone could severely underrepresent the taxa most actively engaged in the EM symbiosis. *Tuber*, a hypogeous ascomycete, and *Tomentella*, a corticioid basidiomycete, are both easily overlooked during fruit body collections. Additionally, *Tuber* demonstrates the necessity of sampling across a wide taxonomic range. Some below-ground studies have used basidiomycete-specific primers to avoid contamination from nonmycorrhizal ascomycetes (Parrent et al. 2006, Landeweert et al. 2003), thus raising the sequencing success rate; however, this study provides an example where the most common genus found on root tips would have been missed using basidiomycete-specific primers.

Here, I also present what may be the first documented case of an EM fungus from the Sarcosomataceae. This family of ascomycetous cup fungi is assumed to be saprobic, though very little is known about its ecology (Hansen and Pfister 2006). The Sarcosomataceae comprise part of a clade sister to the Pyronemataceae (Hansen and Pfister 2006, Pfister et al. 2008), another family of operculate discomycetes once assumed to be entirely saprobic. The Pyronemataceae are now known to contain some EM species (Tedersoo et al. 2006), and ancestral state reconstruction reveals six independent evolutions of the EM life strategy within this family (Hansen et al. 2013). While my

detection of an EM species within the Sarcosomataceae is not definitive, it does indicate that the ecology of Sarcosomataceae merits further study.

Interestingly, the list of species recovered from root tips does not include some putatively ectomycorrhizal species found during concurrent fruit body collection, such as members of the genera *Hysterangium*, *Rhizopogon*, *Scleroderma*, and *Ramaria* (Trappe et al. unpublished data). While I advocate the study of the EM fungal community using below-ground techniques, surveys of fruit bodies are clearly still necessary, both for sampling diversity and for describing the morphology of new taxa.

Most sequences could not be identified down to the species level due to a combination of low sequence similarity and insufficiently annotated GenBank accessions. This illustrates the need for further molecular studies in subtropical North America, particularly because the subtropics and tropics provide excellent opportunities to study the effects of host density versus host richness.

We must be cautious in interpreting genus-level data, since ecological variability exists within any given genus. However, species within the same genus usually share macroscopic features such as hyphal exploration type (Agerer 2001) and fruit body morphology, and are probably more biochemically similar to each other than they are to species from a separate, independent evolution of the EM symbiosis. In the absence of species data, analysis at a higher taxonomic level can reveal broad-scale ecological patterns and highlight possible future directions for research.

The potential effects of host preference are particularly relevant for the already at-risk Mexican oak forests. Some land-use practices have been demonstrated to cause a shift in community composition from oak to pine dominance, and a disproportionately high representation of pines leads to decreased soil fertility and an understory of depauperate species richness (García-Barrios and González-Espinosa 2004). This shift also has socio-economic implications, because pine makes inferior firewood and is less desirable as a commercial product. For these reasons, the preservation of Mexican oak forests will likely become a major concern in the near future. EM fungi are a critical and understudied factor influencing the outcome of competition between different host trees, and a more complete understanding of EM community assembly may aid future efforts at forest management.

Table 3. Nine sampling sites across northeast Mexico. Exact host species information is not known for some sites.

Collection site	State	Lat (N)	Long (W)	Elevation	Description	Dominant host trees
mining road	Nuevo Leon	26° 06'	99° 57'	1060 m	dry oak forest	<i>Quercus</i>
El Barro uphill	Nuevo Leon	25° 31'	100° 13'	600 m	oak forest	<i>Quercus</i>
El Barro downhill	Nuevo Leon	25° 31'	100° 13'	600 m	oak forest	<i>Quercus</i>
La Estanzuela high	Nuevo Leon	25° 31'	100° 17'	1990 m	mixed oak/pine	<i>Q. polymorpha</i> , others
La Estanzuela low	Nuevo Leon	25° 32'	100° 16'	770 m	oak forest	<i>Q. polymorpha</i> , other <i>Quercus</i>
El Madrono	Tamaulipas	23° 36'	99° 14'	1460 m	oak woodland	<i>Q. canbyi</i> , <i>Q. polymorpha</i> , <i>Q. laeta</i>
Las Mulas high	Tamaulipas	23° 37'	99° 14'	1590 m	shrub oak woodland	<i>Q. intricata</i>
Las Mulas low	Tamaulipas	23° 37'	99° 15'	1520 m	oak woodland	<i>Q. canbyi</i> , <i>Q. polymorpha</i>
Miquihuaua	Tamaulipas	23° 36'	99° 42'	2610 m	mixed oak/pine/madron	<i>Q. affinis</i> , <i>P. montezumae</i> , <i>P. teocote</i>

Table 4. Ectomycorrhizal taxa ranked by abundance.

<u>Order</u>	<u>Family</u>	<u>Genus</u>
Pezizales - 57 total	35 Tuberaceae	35 Tuber
	11 Pezizaceae	6 Peziza
		4 Pachyphloeus
		1 Genus Unknown
	7 Pyronemataceae	4 Genea
		2 Humaria
		1 Genus Unknown
	1 Sarcosomataceae	
	3 Family Unknown	
	35 Thelephoraceae	34 Tomentella
Thelephorales - 35 total		1 Thelephora
Agaricales - 25 total	23 Cortinariaceae	18 Inocybe
		4 Cortinarius
		1 Hebeloma
	2 Tricholomataceae	1 Laccaria
		1 Mycena
Sebacinales - 18 total	18 Sebacinaceae	17 Sebacina
		1 Tremelloscypha
Russulales - 19 total	19 Russulaceae	10 Russula
		9 Lactarius
Cantharellales - 6 total	6 Clavulinaceae	6 Clavulina
Dothideomycetes - 6 total		6 Cenococcum
Boletales - 4 total	2 Sclerodermataceae	2 Scleroderma
	2 Boletaceae	1 Strobilomyces
		1 Genus Unknown
Atheliales - 2 total	2 Atheliaceae	2 Piloderma

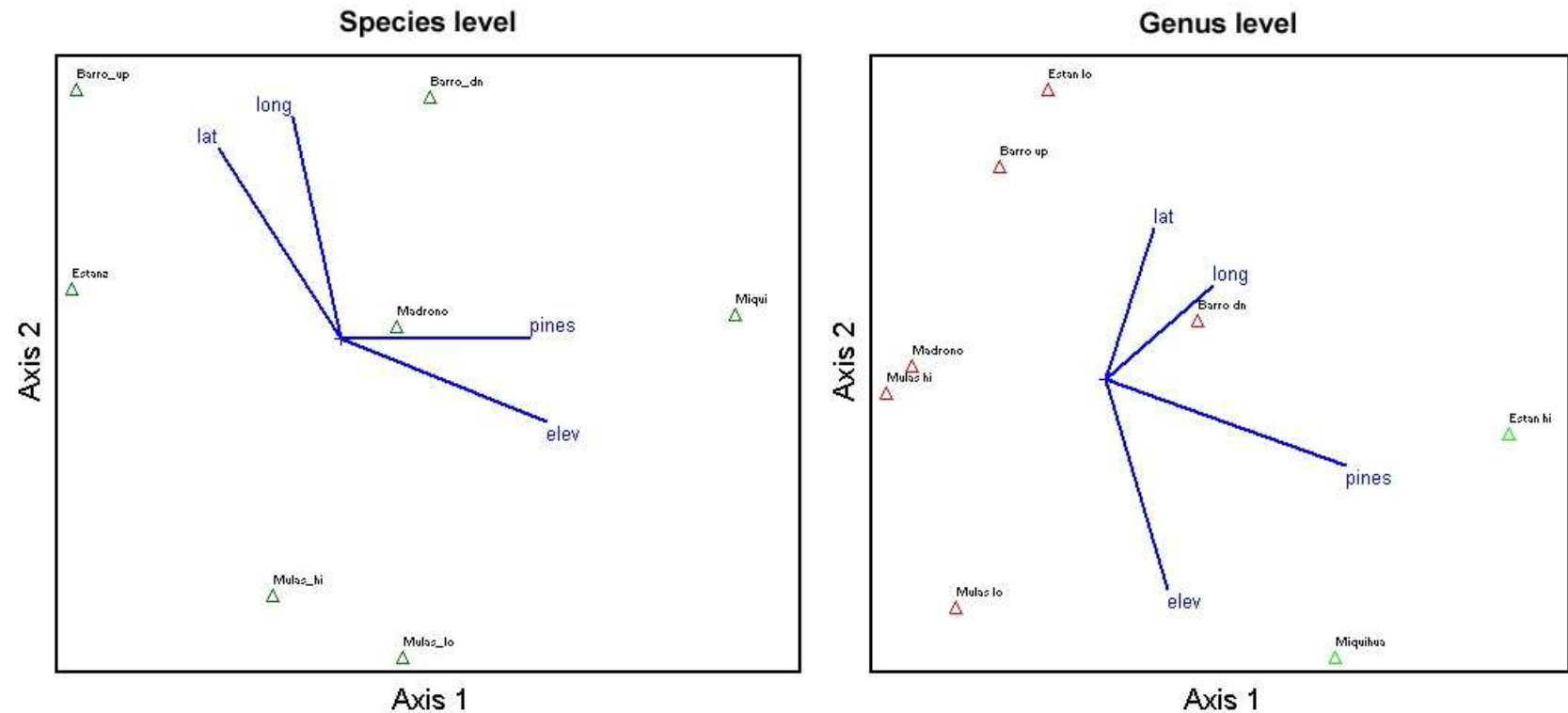


Figure 13. NMS ordinations for EM fungal abundances at sites in northeast Mexico with abundances tallied at two phylogenetic levels: species and genus. At the species level, the high-elevation Estanzuela site did not share enough in common with other sites to be included in the analysis, so this site appears only in the genus-level ordination. The sites in the genus-level ordination are also presented at a cluster level of two groups, with one group depicted in green and the other in red. Vectors show correlations of environmental variables.

Figure 14. NMS ordination for three regions with vectors showing correlations of environmental variables. Sites are grouped at a cluster level of 3 in the Axes 1 versus 3 graph (left) and at a cluster level of 4 in the Axes 2 versus 3 graph (right).

4. Proximity to mature trees enhances availability of ectomycorrhizal fungi for seedlings of *Dicymbe corymbosa* (Caesalpiniaceae) in Guyana

4.1 Introduction

Neotropical lowland rain forests are characterized by high alpha diversity of tree species and relatively few ectomycorrhizal host plants. Monodominance is rare, and where it does occur, ecologists struggle to determine how it develops and persists (e.g. Lopez and Kursar 2007, Fonty et al. 2011, Peh et al. 2011). Often, monodominance can be attributed to temporary successional seres or harsh localized environmental conditions such as inundated soils (Hart 1990, Richards 1996). For a species growing on well-drained soil to attain persistent monodominance, it may require a suite of unique traits to exclude other species from establishing within the stand (Torti et al. 2001). *Dicymbe corymbosa* (Caesalpiniaceae) is perhaps the most dramatic example of such a species.

Native to the Guiana Shield region of northeast South America, *D. corymbosa* forms monodominant stands wherein it can account for up the 95% of the basal area (Henkel et al. 2002, Henkel 2003). These stands occur on well-drained upland sites, embedded in a matrix of high-diversity mixed forests. *Dicymbe corymbosa* expresses a number of unusual characteristics which may contribute to the establishment and persistence of monodominant stands, including reiterative growth and coppicing (Woolley et al. 2008), mast fruiting and extended survivorship among established

seedlings (Henkel et al. 2005), root mounds that act as litter catches (Henkel 2003), and perhaps most interesting: the ability to associate with ectomycorrhizal (EM) fungi in addition to the arbuscular mycorrhizal (AM) fungi found on the roots of the majority of neotropical trees (Henkel et al. 2002, McGuire et al. 2008).

Most temperate forests are dominated by one or more EM-associated tree taxa, such as Pinaceae and Fagaceae. In these forests, the dominant canopy species controls the mycorrhizal assemblage in the soil, which can affect the ability of seedlings to locate suitable symbionts (Chapter 1). Seedlings may preferentially associate with common mycorrhizal networks (CMNs), because these mature fungi are receiving ample carbon from canopy trees, and thus may be able to provide low-cost nutrients to seedlings (Simard and Durall 2004). CMNs have been shown to improve survivorship of seedlings by compensating for the competitive effects of adult conspecifics (Booth and Hoeksema 2010). In *D. corymbosa* stands, seedlings with access to CMNs outperform seedlings without access -- both in terms of growth and survivorship -- despite no significant difference in root-tip colonization (McGuire 2007a). In this context, CMNs may counteract Janzen-Connell effects (Janzen 1970, Connell 1971a, Connell 1971b) and contribute to the ability of *D. corymbosa* to form monodominant stands in the tropics.

A previous study found that 1-year-old seedlings had lower survivorship and less mycorrhization in mixed forest than in monodominant stands (McGuire 2007b). This suggests that a single adult *D. corymbosa* provides an insufficient reservoir of EM fungi,

which hinders the establishment of its seedlings. However, it is not clear that the relationship is causative; both seedling survivorship and mycorrhization may have been responding to a third factor, such as the chemical composition of litter deposited by non-*Dicymbe* trees. Or low mycorrhization may have been a result, rather than a cause, of poor seedling health and imminent mortality.

Here, I examine the diversity of EM fungi associated with seedling roots and available in the rhizosphere soil, in order to assess how species richness and community composition may affect seedling recruitment. In mixed forest where only one adult host tree is present, I predict a distance-dependent effect on the availability of EM fungi for seedlings, and I expect the lack of access to EM fungi to be reflected in the isotopic ratios of the seedlings.

4.2 Methods

4.2.1 Study Site and Experimental Design

This study was conducted in the Pakaraima Mountain Range of central Guyana, near the Upper Potaro river basin (5°18'N, 59°54'W; elevation 720m). I sampled from long-term plots established in monodominant *D. corymbosa* forest (Henkel 2003) as well as transition zones into adjacent mixed forest.

At three locations in the transition zone between monodominant forest and mixed AM-dominated forest, I identified the farthest away reproductive-age individual of *D. corymbosa* and drew a transect away from the base of the tree in the direction of the

mixed forest. I then found three "near" seedlings (at the base of the parent tree's root mound), three "medium" distance seedlings (approx. 7m along the transect), and three "far" seedlings (approx. 15m along the transect). For comparison, two transects were delineated inside monodominant stands and seedlings were chosen following the same procedure.

After seedlings were examined to determine their approximate age, they were dug up and the dirt rinsed from the roots. I sampled either all EM root tips (for mixed forest seedlings) or a representative selection of all EM morphotypes (for stand seedlings). Soil samples were collected from the rhizospheres of all nine seedlings, with an additional tenth sample taken at a distance of ~20m along each transect. Leaves were collected from "far" seedlings in mixed forest, and from adults and "near" seedlings in monodominant stands. Root tips and soil samples were silica-dried to preserve them for transport.

DNA was extracted from root tips following the Extract-N-Amp protocol (Sigma-Aldrich, Inc.) with individual root tips in 20µl extraction solution, and the internal transcribed spacer (ITS) region of ribosomal DNA was amplified via polymerase chain reaction using the forward primer ITS1-F (Gardes and Bruns 1993) and either ITS4 or ITS2 as a reverse primer (White et al. 1990). This region was sequenced using BigDye v3.1 and a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA). For identification, root-tip sequences were compared against an extensive database of ITS

sequences from EM fruit bodies and adult root-tips collected in Guyana (M.E. Smith, unpublished) using ViroBLAST (Deng et al. 2007). Sequences were submitted to Genbank under accession numbers XXXXXXXX-XXXXXXX.

DNA extractions from soil samples were performed with a PowerSoil Extraction Kit (Mo Bio). The ITS region was amplified with tagged primers, the amplicons pooled, and high-throughput pyrosequencing was performed on a Roche 454 GS FLX (454 Life Sciences).

4.2.2 Data Analysis

To compare the community composition of seedling-associated EM fungi within and outside of monodominant stands, PC-Ord version 5 (McCune and Mefford 1999) was used to perform nonmetric multidimensional scaling (NMS). A two-dimensional solution was selected based on the stress results of a stepdown procedure.

The high-throughput soil sequences were denoised in QIIME version 1.8 (Caporaso et al. 2010) using the `denoise_wrapper.py` function (Reeder and Knight 2010). These data were clustered with a modified version of the `pick_open_reference_otus.py` pipeline, using blast for prefiltering at 85% similarity and as the clustering method for the initial round of OTU picking. The centroid-based method `uclust` (Edgar 2010) was then used to recover de novo OTUs from those sequences that failed to match the

reference data. These de novo OTUs were identified by comparison against a subset of the UNITE database, and only EM taxa were retained for analysis.

4.3 Results

While seedlings recruited within monodominant plots yielded ample EM root tips, consistent with previous observations of high colonization rates (McGuire 2007a), my direct sequencing approach was constrained by the very low abundance of EM root tips found on transition-zone seedlings. Because of this, no effect of distance from parent tree was observed, though the EM diversity per seedling was consistently higher at all distances for monodominant transects (Fig. 15). Overall, seedlings in monodominant stands yielded significantly greater species richness per seedling than those in transition zones ($P < 0.001$).

High-throughput sequencing provided a more comprehensive sampling of the EM community available in the soil, recovering close to twice as many species in the monodominant stands (Fig. 15). In mixed forest, EM species richness per soil sample was high near the parent trees and steadily decreased with distance. Taking each transect as a whole, EM species richness was lower in mixed-forest soil than monodominant-stand soil (Fig. 17). The Clavulinaceae, Russulaceae, and Thelephoraceae were well represented in all transects, which is consistent with results obtained from seedling root tips. Of the 61 species found on root tips, 31 were found in multiple

transects or at multiple distances and thus were informative for NMS analysis (Fig. 16). The soil sequencing recovered 105 putatively ectomycorrhizal OTUs, of which 43 were among those found on seedling root tips. The 62 remaining sequences included 26 species known from fruit bodies, 23 OTUs found previously on adult roots, and 13 *de novo* OTUs which belong to EM lineages.

My initial attempt at picking OTUs with the uclust method (Edgar 2010) in QIIME yielded puzzlingly few matches to the reference database and a large number of *de novo* OTUs, which did not make sense given the comprehensive fruit body sampling that produced the reference data. There was also a clear taxonomic bias in how well the clustering method worked, with *Russula* overrepresented and other common taxa, such as *Tomentella*, entirely absent. From this I concluded that centroid-based clustering methods may be too conservative for the highly variable ITS region, so I switched to BLAST for comparison against the reference database. The data remaining after this step are more likely to contain erroneous sequences, so a conservative method such as uclust may be preferable for the recovery of *de novo* OTUs.

4.4 Discussion

Both direct root-tip sequencing and soil sequencing detected low EM diversity in transition zones compared with monodominant stands, suggesting that the low mycorrhization rates of transition-zone seedlings are, indeed, a product of the decreased

availability of EM fungi. Transition-zone seedlings, even those growing directly beside the parent tree, yielded so few root tips that a distant-dependent decrease in EM diversity could not be observed. The diversity within monodominant stands, however, was high enough to demonstrate a definite absence of distant-dependent effects along those transects. This indicates that the high abundance of *D. corymbosa* adults can support enough EM fungi to effectively saturate the soil.

The high-throughput sequences corroborate the idea that, within stands, proximity to an adult tree does not alter the availability of EM fungi in rhizosphere soil. The two soil samples taken at 20m yielded slightly less EM diversity, but this is likely because these samples were not from the rhizosphere of a seedling. It appears that successfully established seedlings boost EM diversity on a very small spatial scale, assumedly by attracting hyphae for mycorrhizal root tip formation. This is an interesting result in the context of McGuire's CMNs theory (2007a), in which *D. corymbosa* seedlings are heavily dependent on parasitizing adults for survival and growth. Since seedlings can persist in the understory for years with very little growth, the amount of carbohydrates and nutrients stolen from the fungi may be negligible while the seedlings wait for a canopy gap to open. However, if it is a significant amount, either the fungi cannot distinguish the roots of parasitic seedlings from those of mutualistic adults, or the adults are actively selecting for fungi that will feed their progeny.

In mixed forest, the diversity of EM fungi in the soil dropped off with distance from the parent tree, as predicted. At approximately 20m, the non-rhizosphere soil samples recovered no EM species for two of the three transects; since pyrosequencing can detect fungi not only from mycelium but from the spore bank as well, this result indicates a profound lack of EM symbionts available to seedlings. The rhizosphere soils yielded more EM species with increasing proximity to the parent tree, though even the samples collected at the base of the adult's root mound contained significantly less EM diversity than any rhizosphere samples from within the monodominant stands.

Apparently, *D. corymbosa* adults that are isolated from conspecifics in AM-associated mixed forest either cannot acquire or cannot maintain the level of EM diversity seen in monodominant stands.

Since stands serve as the source from which outlying trees receive fungal inoculum, it is not surprising to find that the communities are quite similar. While there is some separation between the EM community composition of transition zones and monodominant stands (Fig. 16), the difference is primarily driven by the low diversity found on "far" seedlings from the transition-zone transects. The Clavulinaceae, Russulaceae, and Thelephoraceae were found on multiple seedlings in all transects, which is consistent with their common occurrence on mature *D. corymbosa* roots. Some, but not all, of the taxa within these families behaved predictably based on data from adult trees. For example, *Tomentella* "brown fuzzy" mes348, the most common member

of the Thelephoraceae found in previous studies, was present on multiple seedlings in all transects.

Other species, however, varied in their local abundance. *Tomentella* TH8977 -- a rare species on adult roots -- was absent from monodominant stand transects but found in all three transition zone transects, especially in association with "far" seedlings. This species may be well adapted for spore dispersal and colonization, or poorly adapted for the highly competitive niches inside established stands. I also found examples of the opposite pattern. The genus *Inocybe* was found on seedlings at all distances in the monodominant stands, but only found on one "near" and one "medium" distance seedling in transect three. The soil community analysis supported the absence of *Inocybe* from the rhizosphere in the other two transition zones. *Inocybe* appears to be dispersal-limited and strongly reliant on adult trees to support the fungal individuals also associating with seedlings.

The functional differences driving these patterns of community composition remain unclear, and they may well have an impact on the health of seedlings. An EM species may be common because it provides excellent service to and is therefore preferred by its host; or it may be a comparatively poor mutualist that nonetheless aggressively colonizes root-tips to the exclusion of other, preferred species. If "competition-adapted" is taken to mean the former of these two strategies, then we may infer the dispersal-adapted fungi available in the transition zones are indeed lower

quality mutualists. In this context, EM community composition may affect seedling fitness even in the absence of differences in total species richness or in the availability of access to CMNs.

Curiously, the Boletales were noticeably underrepresented or absent on all seedlings, including those growing within monodominant stands. Boletes comprise approximately 10-20% of the EM diversity on adult roots (Smith et al., unpublished) and are commonly found as fruit bodies. But only three species were recovered from seedling roots, compared to two dozen found on adult roots, and of these three only *Xerocomus* TH8850 could be included in the NMS analysis because the other two did not occur in multiple locations. However, members of the Boletales were detected in the soil samples of all five transects -- including ten species across four genera -- demonstrating that Boletalean spores and/or hyphae were available in the seedlings' rhizospheres. It's unclear whether this lack of association results from a preference for mature canopy trees on the part of the boletes or a dislike of boletes on the part of the seedlings. Boletes may be particularly adept at retaining the photosynthates they receive from adults, or at avoiding root-tips that do not provide a favorable exchange rate of photosynthates for nutrients.

These findings point to several possible mechanisms that might serve to maintain the hyper-diversity of fungi found in association with *D. corymbosa*, including specialization on adults, spore dispersability, and interspecific competitive ability. I also

observed a distant-dependent effect on the availability of EM fungi as well as low diversity overall in mixed forest, both of which support the theory that EM fungi play an important role in counteracting Janzen-Connell effects and thus facilitating the formation of monodominant stands.

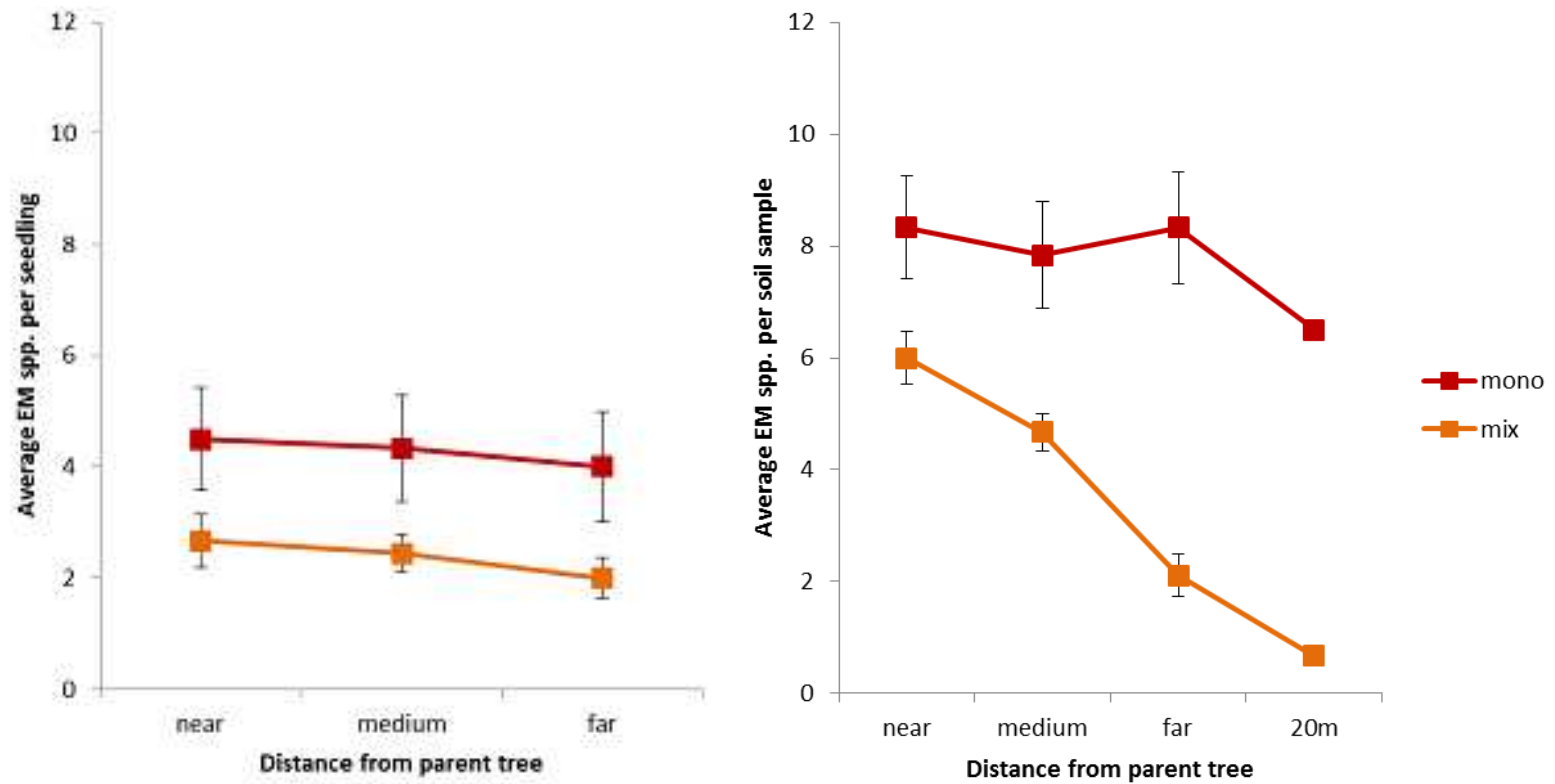


Figure 15. EM species richness per seedling as a function of distance from the parent tree. Three transition zone transects ("mix") and two monodominant stand transects ("mono") were sampled using direct sequencing of EM root tips (left) and 454 sequencing of soil fungi (right). Near, medium, and far samples were taken from seedling rhizospheres; the 20m samples represent non-rhizosphere soil beyond the farthest seedling. Average EM species per seedling.

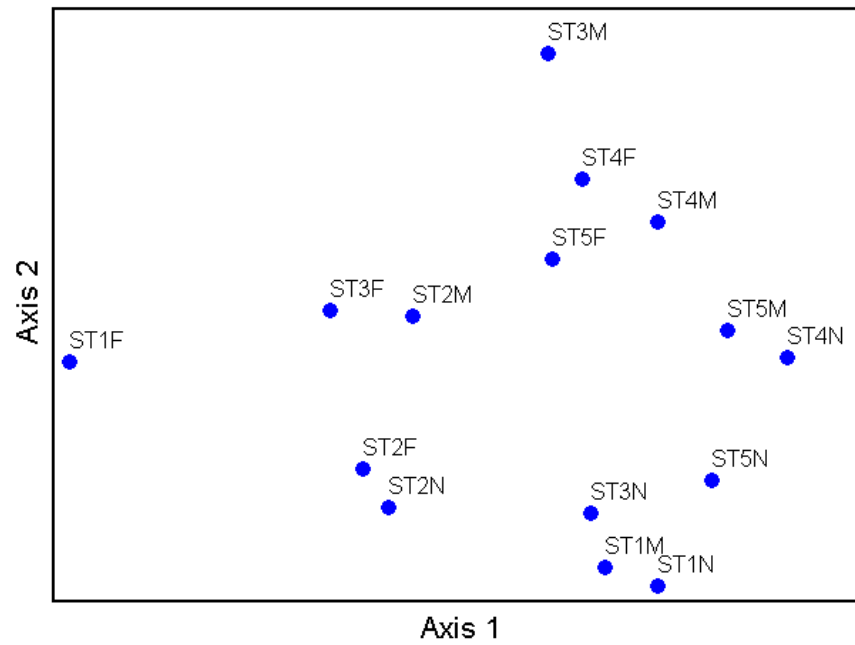


Figure 16. Two-dimensional NMS ordination of fungal communities in the soil. Mixed forest transects (ST1, ST2, ST3) and monodominant stand transects (ST4, ST5) appear somewhat separate. No effect of distance (Near, Medium, Far) on community composition is evident.

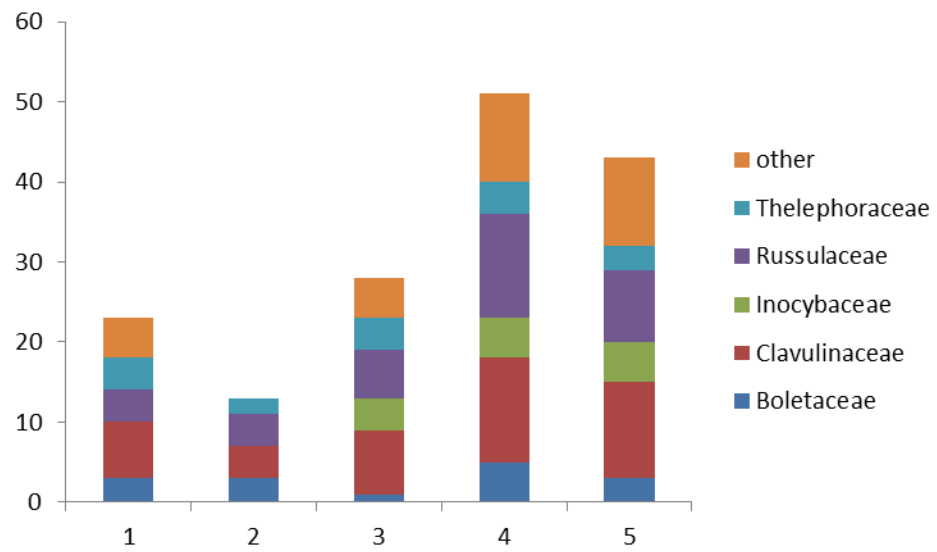


Figure 17. EM species richness in soil, broken down by transect and family.

5. Do seedlings steal carbon and nitrogen from ectomycorrhizal fungi in monodominant stands of *Dicymbe corymbosa*?

5.1 Introduction

An individual ectomycorrhizal (EM) fungus may affect plant community structure by associating with more than individual host. Common mycorrhizal networks (CMNs) are a concurrent association with multiple plants that facilitates the transfer of compounds -- such as carbohydrates, water, and nutrients -- between different host individuals. Myco-heterotrophic (MH) plants such as Indian pipes (*Monotropa uniflora*) and other members of the plant tribe Monotropoideae indirectly parasitize other plants via these CMNs (Trudell et al. 2003). However, the quantity of carbon transferred through CMNs between predominantly photosynthetic species and even between conspecific hosts is still a matter of debate (van der Heijden and Horton 2009).

Partially MH plants produce chlorophyll and photosynthesize, but also rely on mycorrhizal fungi as a source of supplemental carbon. Often, plants that utilize an MH germination strategy will also be partially MH as adults; this includes members of the well-studied Orchidaceae as well as the Pyroleae tribe within the Ericaceae (Tedersoo et al. 2007, Hynson et al. 2009). In other cases, seedlings may tap into prepaid networks established by "nurse" plants not for any nutritive benefit but to improve their drought resistance (Richard et al. 2009).

Stable isotope ratios can indicate whether or not seedlings are receiving nitrogen or carbon from CMNs. Due to isotopic fractionation during synthesis of transfer compounds, EM fungi retain $\delta^{15}\text{N}$ -enriched nitrogen and preferentially transfer $\delta^{15}\text{N}$ -depleted nitrogen to the host tree (Hobbie and Hogberg 2012). The host tree, in contrast, packages $\delta^{13}\text{C}$ into simple sugars, and secondary products such as lignin and lipids are comparatively depleted in heavy carbon (Gleixner et al. 1993). Mycorrhizal fungi receive the simple sugars and thus show a significant isotopic enrichment in $\delta^{13}\text{C}$ relative to host plant foliage (Hobbie et al. 1999).

The EM fungal community's enrichment in heavy isotopes of both carbon and nitrogen is then reflected in the isotopic ratios of myco-heterotrophic plants (Hynson et al. 2009). Entirely MH plants have isotopic ratios similar to those of the fungi they associate with (Trudell et al. 2003), and partially MH plants show $\delta^{13}\text{C}$ enrichment proportional to their carbon uptake (Teder et al. 2007).

Dicymbe corymbosa (Caesalpineaceae) is an ectomycorrhizal legume that forms monodominant stands in the lowland Neotropics. These stands support unique EM fungal communities within a matrix of AM-associated forest characterized by high levels of plant diversity. Once established within a monodominant stand, the seedlings of *D. corymbosa* persist in low-light understory conditions for years (Henkel 2003), which is especially puzzling in the context of Janzen-Connell effects. Partial mycoheterotrophy may be one mechanism that allows for their long-term survivorship.

I predict that *D. corymbosa* seedlings with access to CMNs will show heavy isotopic enrichment similar to that of partially MH plants, indicating that they are receiving nitrogen and some carbon from EM fungi. Seedlings established outside monodominant stands and far away from their parent tree should have limited access to EM fungi, and thus be depleted in heavy isotopes relative to stand seedlings.

5.2 Methods

The study sites used here are near the Upper Potaro river basin in the Pakaraima Mountain Range of central Guyana (5°18'N, 59°54'W; elevation 720m). Samples were taken from long-term research plots within monodominant *D. corymbosa* forest and transition zones into adjacent non-EM mixed forest.

Leaves were collected from transects delineated for a concurrent project (Chapter 4). From monodominant stands, I sampled adult trees and seedlings recruited directly at the base of the root mounds of those adults. From mixed forest, only the farthest seedlings were sampled to minimize the availability of EM fungi. When possible, younger leaves were selected to minimize the quantity of epifoliar growth. Leaves were dried with silica to preserve isotopic ratios during transport.

Several small pieces were broken off each dried leaf and pulverized in a ball mill. For each sample, 2.5-4 mg of this powder was run through a Carlo Erba Elemental Analyzer (CE Elantech, Inc, Lakewood, NJ) coupled to a Finnigan MAT Delta Plus XL

continuous flow mass spectrometer (Thermo Fisher Scientific, Inc). This process determined the carbon and nitrogen content as a percentage of dry mass as well as stable isotope ratios.

5.3 Results

Averages of %N, %C, and stable isotopic ratios are presented for each sample group (Table 5). Stand seedlings were enriched in $\delta^{15}\text{N}$ compared to stand adults, and mixed-forest seedlings had an intermediate value but were not significantly different from either of the other sample groups. Mixed-forest seedlings were, however, depleted in $\delta^{13}\text{C}$ compared to both stand seedlings and stand adults.

5.4 Discussion

Stand seedlings were significantly enriched in foliar $\delta^{15}\text{N}$ compared to adults, indicating that they are behaving as partial myco-heterotrophs, but the data for mixed-forest seedlings are inconclusive. Both stand seedlings and adults showed $\delta^{13}\text{C}$ enrichment compared to mixed-forest seedlings, which suggests that seedlings with access to EM fungi are donating carbon to those fungi in the same proportion as adults. This is surprising, since light availability is much higher for canopy trees than for understory seedlings, and low light conditions encourage transfer of carbon from fungi to partially myco-heterotrophic plants (Zimmer et al. 2007). Even if *D. corymbosa* seedlings are unable to "steal" carbon from adults via CMNs, I would expect them to

donate very little carbon to the fungi -- especially given that $\delta^{15}\text{N}$ -enrichment is a sign of "stealing" nitrogen from the fungi, instead of receiving $\delta^{15}\text{N}$ -depleted transfer compounds.

The self-contradictory stable isotope results suggest that the CMN interactions among adults, seedlings, and EM fungi may be more complicated than originally thought. A more comprehensive isotope study, including data from fruit bodies and root tips, would help to illuminate the role of CMNs in seedling recruitment. One possible explanation for the stable isotope data presented here is that the quantity of carbon and nitrogen transfer and degree of isotope fractionation depend on which EM taxa predominate on a small spatial scale. These traits are likely to be a component of the functional diversity that allows so many EM species to persist in association with a single species of host.

Table 5. Stable isotope results for carbon and nitrogen contradict one and other. Letters indicate statistical significance.

Leaf origin	%N	$\delta^{15}\text{N}$ (‰) *	%C	$\delta^{13}\text{C}$ (‰) **
mixed forest seedlings	1.64 ^a	2.12 ^{a,b}	52.1	-35.47 ^a
stand seedlings	2.31 ^b	2.33 ^b	52.6	-27.94 ^b
stand adults	1.55 ^a	1.58 ^a	52.34	-29.17 ^b

* relative to atmospheric N₂

** relative to V-PDB standard

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Biography

Gwendolyn C. Williams was born on June 10th, 1984 in New Haven, CT. She attended University of Chicago, where she received a Metcalf Fellowship to intern in the mycological herbarium at the Field Museum. She then completed an honors thesis on arbuscular mycorrhizal fungi under the mentorship of Dr. Gregory Mueller. She graduated in 2006 with general honors, a BS in Geophysical Sciences, and a BA with honors in the Ecology and Evolution Specialization of Biological Sciences. At Duke University, she received a James B. Duke Fellowship for outstanding new scholars. The Forest History Society awarded her the FK Weyerhaeuser Fellowship in support of her Duke Forest research projects.